Surfactant Uptake by Alveolar Cells: Factors affecting lipid uptake in vivo and in vitro

Surfactant opname door alveolaire cellen: factoren die de lipid opname in vivo en in vitro beïnvloeden

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CHAPTER 1

Surfactant Metabolism by Alveolar Cells: factors affecting lipid uptake *in vivo* and *in vitro*

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Modified version accepted for publication

Introduction

Kurt von Neergaard[1] was the first to draw attention to the role of surface tension within the lung. In 1929, he demonstrated that the pressure needed to inflate a fluid-filled lung with fluid was less than approximately ⅓ to ¼ of the pressure necessary to inflate an air-filled lung with the same volume of air (Fig. 1[1]). From these experiments he concluded that about two-thirds of the retractive forces are due to surface tension phenomena acting at the air-liquid interface within the lung; and accordingly this surface tension at the alveolar level is reduced by the presence of a surface active agent with a low surface tension to allow normal breathing. Unfortunately, these findings were published in the German language only and for approximately 25 years they remained practically unnoticed by other scientists in the field. The next description of the presence of a surface active agent within the lung was in 1954 by Macklin who described a thin aqueous mucoid film, formed by granular pneumocytes, on the alveolar wall and which moved constantly towards bronchioles and phagocytic pneumocytes[2]. The following year, it was noted by Pattle and colleagues, that the foam and bubbles from lung edema and healthy lung cut had a remarkable stability; they concluded that these bubbles consisted of a surface active agent, able to lower surface tension towards zero[3].
In 1957, Clements used the Wilhelmy balance to demonstrate that the surface tension derived from the alveolar lining fluid of the lung was not a constant value; with a large surface, surface tension was high, but when the surface area was decreased, surface tension fell to values near zero[4].

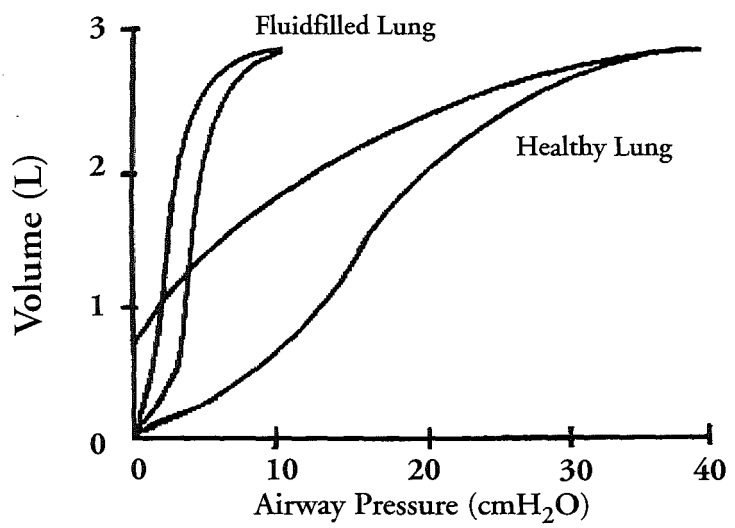

Figure 1. Pressure volume diagram of a healthy air-filled lung and an ARDS lung. In ARDS higher pressures are required to expand the lung compared to a healthy lung due to the high surface tension at the air liquid interface in the alveoli. Which is caused by surfactant inactivity. Adapted from von Neergaard[1].

Avery and Mead made the first steps towards extensive research on this surface active agent, called pulmonary surfactant, by demonstrating higher surface tension in very small premature infants and infants dying of respiratory failure due to hyaline membrane disease[5]. Even today,

almost half a century later, small steps are still being made toward a better understanding of this surface active agent.

Pulmonary surfactant

This pulmonary surfactant, lining the alveolar surface, is a complex of lipids and proteins produced in the alveolar type II cells and secreted into the alveolar space.

Lipids
The lipid composition is generally the same in both compartments[6, 7]. Most of the lipids are phospholipids (80-90%), and in decreasing order of content are cholesterol, triacylglycerol and free fatty acids[7] (Fig. 2).
Phosphatidylcholine (PC) compromises most of the phospholidids (70-80%), of which approximately 50% is disaturated (DPPC)[6, 8].
This subgroup of PC is an unusual species, with palmatic acid at both the 1-position and 2-position rather than a saturated fatty acid at the 1-position and unsaturated fatty acid at the 2-position of the diacylglycerolphospholipid found in most mammalian tissues; although not specific for surfactant (because it is also found in other tissues) it compromises a very high percentage of the surfactant phospholipids.
Already in early fetal gestation, about 20% of the total amount of PC retrieved from the lung is DPPC[9]. This DPPC is the main surface tension lowering phospholipid in the lung. Although only a small fraction of the extracellular DPPC is necessary to cover the alveolar wall throughout the lung with a monolayer (as calculated by Wright and Clements[10]) its pool size is tightly regulated. For example, decreases in the amount of DPPC on the short term due to an abnormal nutritional state such as fasting[11], fatty acid deficiency[12] or choline deficiency[13], are replenished rapidly by adaptation mechanisms[14].

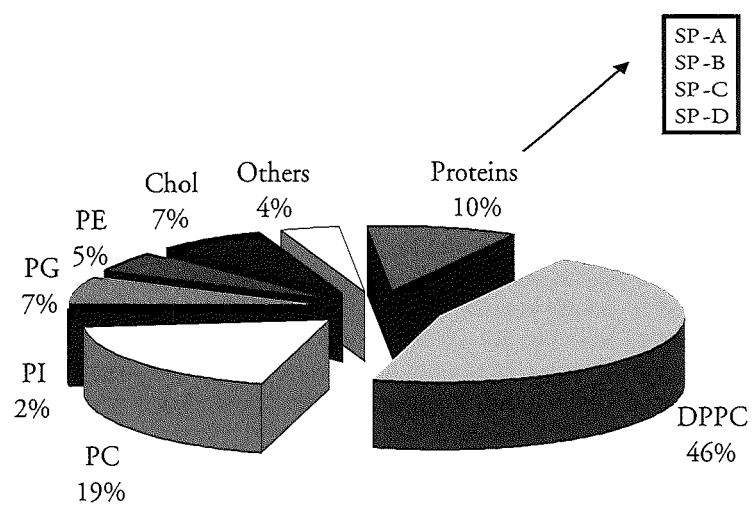

Figure 2. Schematic overview of surfactant composition.
PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; Chol, cholesterol.

Next, all mammalian pulmonary surfactants have been shown to contain significant amounts of phosphatidylglycerol (PG) (7-18%) and phosphatidylinositol (PI) (2-4%)[15], suggesting a specific role of these acidic phospholipids. In adult mammalians, PG is the second major lipid component next to PC, comprising approximately 5-10% of total surfactant phospholipids in humans[7, 16-18] and rats[6, 19]. However, in preterm fetal lungs the PG component in surfactant is extremely low, although its relative absence is compensated by an increased amount of PI[8, 20-22] in the surfactant compartments.
When DPPC is mixed with PG or PI, adsorption of the lipids in the monolayer is enhanced, indicating that these negatively charged lipids may play an important role in the surface tension lowering activity of surfactant. For PG, this enhanced adsorption may be caused by a specific interaction between PG and SP-B[23, 24].
Finally, the remaining phospholipids consist mainly of phosphatidyl-ethanolamine (PE; 2-3%) and some other minor phospholipids, whereas the total surfactant is completed by cholesterol[15].

Proteins
Pulmonary surfactant contains at least four surfactant proteins (SP), SP-A, SP-B, SP-C and SP-D. Of these proteins, SP-A and SP-D are hydrophilic proteins and SP-B and SP-C are hydrophobic.
SP-A has been extensively studied and although its role is not yet completely clear, it is suggested that it plays an important role in regulating surfactant function via binding to phospholipids[25, 26], modifying phospholipid structure to tubular myeline[27, 28], maintaining the surface properties of surfactant[29], regulating secretion and clearance of surfactant[30-37], regulation of alveolar macrophage function[38], as well as a possible role in the immunological properties of surfactant[38, 39].
SP-B and SP-C are two hydrophobic proteins that are known to play an important role in the formation of a stable lipid monolayer. Especially SP-B has been shown to be essential for normal surfactant function, lowering surface tension[40, 41]; absence of SP-B at birth leads to death caused by respiratory insufficiency[42, 43], and conditional knockout of SP-B in adult animals leads to respiratory failure[44]. In addition SP-B is also suggested to play a role in the protection of the surfactant system against endotoxin-induced lung inflammation by enhancing surfactant function, resulting in reduced lung injury, decreased influx of inflammatory cells, and lower cytokine levels[45].
SP-C also enhances the surface active properties of surfactant[40, 41, 46-48]. Although (unlike SP-B) its absence at birth is not lethal, it does result in decreased stability of surfactant at low olumes despite the fact that surfactant pool sizes and lung morphology were similar in wild type and SP-C knockout mice[49]. Another function of SP-C is to increase the resistance of surfactant against inactivation by plasma proteins[50]. On the other hand, elevated expression of SP-C is thought to be related to cytotoxicity and, ultimately, altered lung development[51]. Though SP-D has been suggested to be the fourth surfactant protein, this protein is not only found within the lung but also in other organ systems and its specific contribution with regard to surfactant is not completely clear; however, several studies have suggested that, together with SP-A, it has an immunomodulatory role in the lung[39, 52-55].

Metabolism

The presence of surfactant within the alveolus is the result of a complex system of production, secretion, insertion into the lipid monolayer and turn-over, uptake and recycling (Fig. 3).

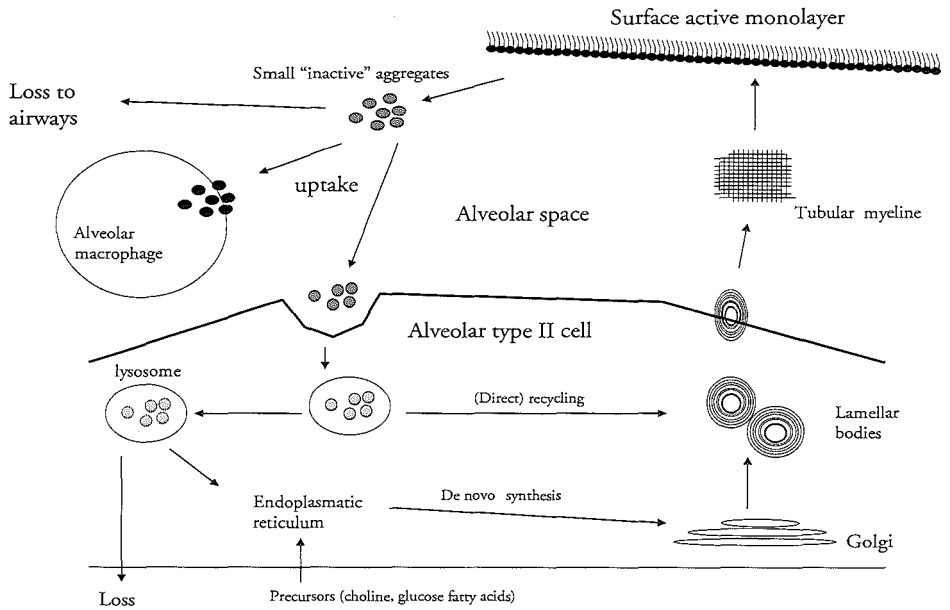

Figure 3. Diagram showing surfactant metabolism.

Production and secretion

Surfactant phospholipids are produced by alveolar type II cells which comprise only 15% of the total number of cells in the lung[56-58]. The *de novo* synthesis of surfactant is thought to be relatively slow, especially in newborn animals[59, 60] as well as in humans as demonstrated by Bunt et al. using stable isotopes[61]. Bunt and colleagues also demonstrated that the use of prenatal corticosteroids increased surfactant synthesis in the preterm infant[62] as well as in very premature baboons[63]. Therefore, most of the surface-active surfactant is produced by recycling. Martini and co-workers demonstrated that approximately 50-90% of the PC in surfactant is recycled depending on age and species, whereby with increasing age the contribution of recycling decreases[64]. The surfactant lipids are synthesized in the endoplasmatic reticulum and then stored in lamellar bodies[65, 66]. In these lamellar bodies surfactant specific proteins A, B and C are already present[67]; however, the content of SP-A is extremely low (1%) suggesting that the SP-A present in the alveolar space might be derived from parts other than lamellar bodies. When the alveolar type II cell is stimulated, intracellular effectors diffuse and activate the movement of the lamellar bodies to the apical plasma membrane of the alveolar type II cells, and the content of the lamellar bodies is secreted into the alveolar space by a process of regulated exocytosis[58, 68, 69] (for more details on the regulation of secretion see[56, 70, 71]).

After excretion into the alveolar space the lamellar bodies unravel and form tubular myeline after association with SP-A[28, 72]. Subsequently, the material of the lamellar bodies is absorbed/ inserted into the lipid monolayer. This tubular myelin is most likely the immediate precursor for lipids inserted into the monolayer; however it should be noted that SP-A knockout mice do not produce normal tubular myelin and the structure and in vitro properties of surfactant have changed. However, the in vivo function of surfactant in SP-A knockout mice has not changed, and thus tubular myeline is not essential for normal lung function[73].

Turnover of surfactant

During respiration the surfactant in the lipid monolayer is turned over from large surface active aggregates into small inactive surfactant aggregates[74-76]. However, little is known about the exact mechanism of this turnover. These small aggregates are not surface active and are removed from the alveolar space to be reutilized or recycled to ensure the presence of surface active aggregates in the lipid monolayer.

Uptake/removal of small aggregates

The turned-over, inactivated surfactant is mainly cleared from the alveolar space by uptake by alveolar type II cells and alveolar macrophages. However, their relative contribution to the uptake of surfactant lipids remains obscure and is dependent on several factors. *In vitro* studies suggested a major role for alveolar macrophages[77], whereas *in vivo* experiments suggested an equal contribution or even a major role for alveolar type II cells in the clearance of surfactant[78-80].

Because of the need of recycling, as suggested previously, re-uptake of surfactant by alveolar type II cells is essential and possibly a crucial factor in the surfactant metabolism. Unfortunately, little is known about the regulation and mechanisms of removal of surfactant from the alveolar space by alveolar type II cells and alveolar macrophages.

This uptake of surfactant lipids is thought to take place (at least in part) via a coated-pit pathway[81-83]. More recently, it was demonstrated that all surfactant phospholipids are internalized via the same pathway by alveolar macrophages and alveolar type II cells, though alveolar cells have a higher affinity for negatively charged phospholipids[84]. In addition, the surfactant proteins are known to affect the uptake, especially SP-A[34, 36, 80, 85, 86].

Measuring the uptake

Most studies on surfactant metabolism, especially those focused on the uptake of surfactant lipids by alveolar type II cells and alveolar macrophages, used radioactive labeled DPPC to measure the uptake. In addition, most studies were performed in an *in vitro* setting, whereas the *in vivo* studies mainly focused on alveolar macrophages. Unfortunately, the use of radioactivity does not discriminate between uptake or intracellular presence of the label and association with the outer cell membrane or adherence. In addition, one cannot specify the specific cells involved in the "uptake" as whole lung tissue is tested on radioactivity.

Recently, we described a method using fluorescent labeled liposomes to study the uptake of surfactant-like liposomes both *in vivo* and *in vitro*, in both alveolar type II cells and alveolar macrophages. Our method mimics the small aggregates of surfactant, as these are the surfactant aggregates generally thought to be removed from the alveolar space. In addition, using confocal laser microscopy it can be demonstrated that the fluorescent labeled liposomes are indeed intracellular located rather than stuck to the outer cell membrane.

More interestingly, in our method it is not DPPC that is labeled but PE, a minor component of surfactant as a part of liposomes consisting of the main lipid components of surfactant, providing the opportunity to study the role of the main lipid components in the regulation of the uptake of surfactant.

One of the advantages of the use of fluorescent labeled liposomes is the possibility to focus on one particular cell type; with the use of specific fluorescent labeled antibodies one can discriminate between different cell types and study their relative contribution or role in the

removal of surfactant lipids and possible mutual regulation of the uptake; even more specific, one can determine whether all cells or a subpopulation of cells is involved in the uptake. Another important advantage of the method described by our group is that it allows to measure uptake by alveolar type II cells and alveolar macrophages both *in vivo* and *in vitro*. We believe we were the first to demonstrate that *in vivo* a significantly small percentage of the alveolar type II cells is involved in the uptake in comparison to *in vitro*; this indicates the need to study uptake both *in vitro* and *in vivo* (Fig. 4).

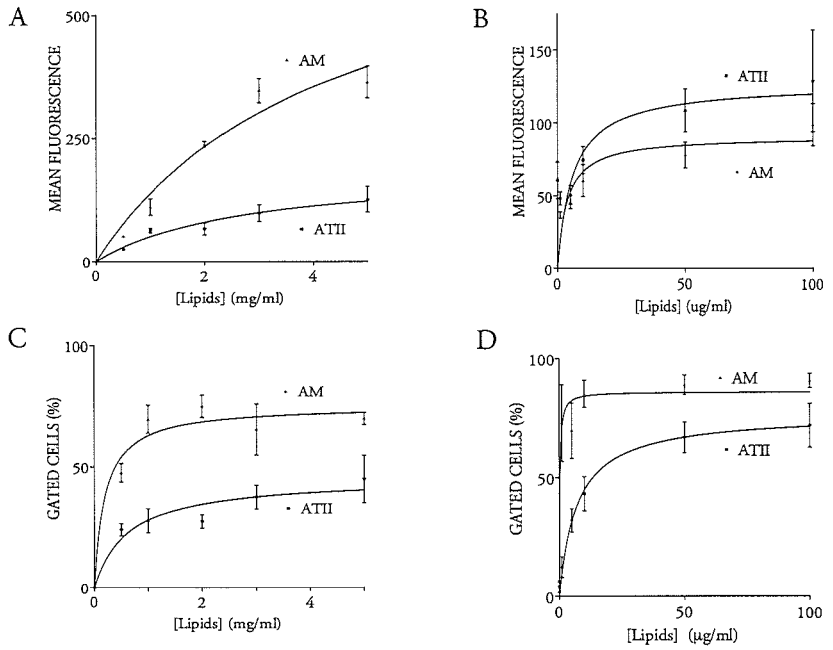

Figure 4. Differences in uptake in vivo and in vitro.
Cell-associated fluorescence as a measure for the uptake of surfactant-like liposomes was determined for different concentrations of labeled liposomes both *in vitro* (*A, C*) and *in vivo* (*B, D*). Additionally the percentage cells involved in the uptake was determined. Data derived from Poelma et al. 2002 . ATII, alveolar type II cells; AM, alveolar macrophages.

Effect of lipid composition on uptake
The composition of surfactant is largely similar across different species, including humans[87, 88]; however, small differences in the relative concentrations of the individual lipids are observed which are also related to age (Table 1). For example, fetal or neonatal surfactant contains a larger percentage of phosphatidylinositol and less phosphatidylglycerol, whereas adult surfactant contains more phosphatidylglycerol than phosphatidylinositol[15]. In addition, neonates have been shown to rely more on recycling and thus uptake than adults[59, 64], which suggests a possible effect/role of the lipid composition on the uptake. Moreover, severe lung injury (initiated by a wide variety of causes) is known to be related to alternations in lipid composition of surfactant[89, 90], which could also contribute to a decreased surfactant function, implying an effect of the different surfactant lipids on the uptake. However, it remains unknown how these alternations in composition are related to the disease.

Table 1. *Breakdown of lipid composition of surfactant, in percentage*

	Phospholipid composition (%) [% distaturated]					
	PC	PG	PI	PS	PE	Cholesterol
Mouse	72.3	18.1			1.9	9.7
Rat	82.3 [49.3]	7.5 [32.3]	1.8 [2.2]	0.1	5.1	7.1
Rabbit (neonatal)	80.6 [52.8]	4.5	6.8		4.4	
Rabbit (adult)	80.6 [52.8]	7.2 [38.7]	4 [2.5]	1.9	4.4	
Human	80.5 [47.7]	11.5	2.5		12.3	7.3
Bovine	79.2 [49.9]	11.3 [33.3]	3.5	3		

PC, Phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phoshatidylserine; PE, phosphatidylethanolamine. Data derived from Veldhuizen et al. 1998 [15], Akino et al. 1992 [127]

Bates et al. and Chander et al. were the first to report on the influence of the individual surfactant lipids on the uptake, demonstrating that radio-active labeled PG was cleared more rapidly by alveolar type II cells *in vitro*[91, 92]. This increased uptake *in vitro* of PG was also demonstrated for alveolar macrophages in comparison to the uptake of DPPC[93].
More recently, we demonstrated a common pathway for the uptake of surfactant lipids by both cell types *in vitro*[84]. It was shown that a significantly lower percentage of alveolar type II cells than alveolar macrophages is involved in the uptake of DPPC (29% versus 72%, respectively), whereas the number of cells involved in the uptake of PG is approximately the same. The presence of a possible phospholipid receptor would simplify the explanation of these results. A different distribution of this phospholipid receptor on alveolar macrophages and type II cells might be the reason for the difference in the percentage of cells involved in the uptake. The uptake of DPPC requires more receptors than the uptake of for instance PG; or, more generally, more negatively charged phospholipids are taken up more easily than neutrally charged phospholipids. A higher receptor density on alveolar macrophages than on alveolar type II cells, and the presence of several subpopulations of type II cells with different receptor densities would explain the lower percentage of alveolar type II cells compared to the alveolar macrophages.
These results with regard to the role of individual surfactant lipids indicate that, besides surfactant proteins, the phospholipid composition of the small aggregates affects the surfactant metabolism. However, these studies were performed *in vitro* whereas significant differences have been demonstrated with regard to the uptake of surfactant-like liposomes by alveolar cells between *in vivo* and *in vitro* experiments, and extrapolation of these results to the *in vivo* situation should be done cautiously[94].
The effects of lipid composition *in vivo* was studied by increasing the amount of PG, the second major phospholipid, present in the small aggregates. The incorporation of PG influences the uptake of surfactant-like liposomes by alveolar cells, though the effects on both cell types differ. The uptake of surfactant-like liposomes by alveolar type II cells is hardly affected by different concentrations of PG. More interesting, however, the influence of the intratracheal instillation of PG-containing liposomes on alveolar macrophages is dramatic; in particular, the

number of alveolar macrophages obtained in the lung lavage is influenced by the amount of PG. In addition, an increase in the amount of PG not only reduces the number of alveolar macrophages but this decrease in the number of cells is accompanied by a deterioration in arterial oxygenation. Although PG does not interfere with the function of endogenous surfactant *in vitro*, as was tested, its increase does lead to a reduced surface activity *in vivo*. Moreover, these adverse affects of PG on endogenous surfactant function can be avoided by adding so-called co-factors like calcium, magnesium, etc.
The "fatal" effect of PG on alveolar macrophages, as suggested by our group, is absent *in vitro*, because in that setting the previously mentioned co-factors are already present in the incubation. However, the effects of PG on the uptake of surfactant-like liposomes by alveolar type II cells *in vivo* are completely different from those derived from the *in vitro* experiments, even when co-factors are present. *In vitro*, increased concentrations of PG result in an increased uptake of these liposomes by alveolar type II cells, whereas the uptake of these liposomes by alveolar type II cells *in vivo* is hardly affected by the concentration of PG within the liposomes, irrespective of the presence of the suggested "co-factors". These results underline the presence of 'enviromental' factors that influence the uptake in vivo and thus emphasize the need to study the uptake of lipids and/or surfactant by alveolar type II cells and alveolar macrophages both *in vivo* and *in vitro*.

Effect of surfactant proteins
As previously mentioned, surfactant contains four proteins; SP-A, SP-B, SP-C and SP-D. First, SP-A has been extensively studied and is thought to play several roles within surfactant homeostasis, especially in the regulation of clearance of surfactant from the alveolar space[85].
Next, both SP-B and SP-C are known to be important for the surface active surfactant monolayer[40-44, 95, 96]. On the other hand, regarding the effects on the uptake of surfactant by alveolar cells, SP-B is capable of increasing the uptake of lipids by alveolar cells[36, 97] (Poelma et al., submitted); however, high concentrations of SP-B are required to induce this increase, which raises the question concerning the physiological contribution of SP-B in regulating the uptake of surfactant lipids by alveolar cells. On the other hand, SP-C has a similar function to SP-B with regard to enhancing surface active properties of surfactant[40, 41, 46-48], but it should be noted that SP-C increases the uptake of surfactant-like liposomes at lower concentrations than SP-B. This effect of SP-C is concentration-dependent, with a maximum at 2% SP-C. In addition, the presence of co-factors (such as calcium, etc.) within the liposomes decreasing the possibility of dilution of the endogenous pool is demonstrated to further increase the effect of SP-C. At an incorporation of 1% SP-C the uptake is already increased, but the maximum increase remains at 2% (Poelma et al., submitted). However, the effects of SP-C on the uptake of surfactant-like liposomes is suggested to be suppressed *in vivo* because *in vitro* experiments have shown a much larger effect on the uptake; more specifically, a non-saturable effect[36, 97].
Furthermore, SP-C is known to associate very rapidly with lung tissue and alveolar macrophages[98, 99]. This increased association, even more rapidly than DPPC, might be an explanation for the increased uptake of liposomes containing SP-C. Although other factors, such as the conformation changes observed in liposomes after incorporation of SP-C, may also affect the binding and uptake of these liposomes by alveolar cells, as was suggested by Rice et al.[100].
Finally, the presence of a putative SP-C receptor could also induce an increased uptake. However, because its presence has not yet been demonstrated, further studies are needed.

Effect of surfactant therapy
Currently, exogenous surfactant is increasingly used in the clinic situation mostly in neonates, but the use of exogenous surfactant in adults is now under consideration[101]. However, the administration of exogenous surfactant is known to influence the endogenous surfactant. Most

studies have focused on the effects of exogenous surfactant on the production and/or secretion of DPPC and have produced conflicting results[33, 102-105]. In premature infants with respiratory distress syndrome, treatment with exogenous surfactant was shown to stimulate the synthesis of endogenous surfactant[106]. Little is known about the clearance or uptake of surfactant. Exogenous surfactant is taken up by alveolar type II cells and alveolar macrophages[107-110]; however, the specific effects of exogenous surfactant, that is surface active surfactant on the clearance of non-surface active surfactant, either endogenous or exogenous is unknown. Our group demonstrated significant effects of exogenous surfactant on the clearance of surfactant-like liposomes (unpublished data). Nevertheless, the effect of exogenous surfactant on the uptake shows significant differences between *in vivo* and *in vitro*.

Effect of surfactant protein analogues

As previously mentioned, SP-B is essential for the biophysical properties of pulmonary surfactant, and is thus most appreciated to be present in exogenous surfactant.

The high cost of naturally derived exogenous surfactant increases the demand for a synthetically-produced surfactant. Therefore, synthetic analogues of SP-B based on the known human amino-acid sequence are nowadays tested and have been demonstrated to closely mimic the function of natural surfactant proteins[111]. In addition these SP-B analogues might be optimized because only essential parts of SP-B are reproduced and further developed to increase the efficiency of the SP-B within the exogenous surfactant preparation.

Synthetic SP-B has been developed and closely mimics the function of natural surfactant proteins[111]. The SP-B analogues are based on the 1-25 sequence of the N-terminal site of human SP-B with a modification at position 11; cysteine replaced by alanine (Cys-11>Ala-11)[112, 113]. A mutant SP-B (serine SP-B-1-25) was synthesized with a site-specific substitution of serine for arginine in position 12 and 17 and for lysine in position 16 and 24 of the N-terminal (Fig. 5). A disulfide linked homodimer of these SP-B analogues was formed by oxidizing the monomeric SP-1-25 peptide[112, 113]. The serine-SP-B-1-25 analogues were demonstrated to be less surface active in comparison to the SP-B-1-25 variants. We showed that the less surface active SP-B mimics serine analogues, and reduced the uptake of surfactant-like liposomes by alveolar type II cells when incorporated in the liposomes; on the other hand, the SP-B-1-25 analogues mimic the effect of native SP-B and do not induce any changes in the

SP-B1-25 monomer

Phe-Pro-Ile-Pro-Leu-Pro-Tyr-Cys-Trp-Leu-Ala-Arg-Ala-Leu-Ile-Lys-Arg-Ile-Gln-Ala-Met-Ile-Pro-Lys-Gly

SP-B1-25 serine monomer

Phe-Pro-Ile-Pro-Leu-Pro-Tyr-Cys-Trp-Leu-Ala-Ser-Ala-Leu-Ile-Ser-Ser-Ile-Gln- Ala-Met-Ile-Pro-Ser-Gly

Figure 5: Peptide sequences of the SP-B analogues and their serine mutants. The SP-B1-25 homodimer consists of two SP-B1-25 monomers disulfide-linked at Cys8 (not shown). The fluorescent label was inserted in all peptides at the N-terminus, shown at the left side of the sequence.

uptake of liposomes by alveolar type II cells (unpublished data). With regard to the uptake of these liposomes with SP-B analogues incorporated by alveolar macrophages, our group demonstrated that the surface active SP-B analogues influence the uptake by alveolar macrophages (unpublished data).
It was also shown that SP-C enhances the surface tension lowering properties of surfactant; therefore, surfactant preparations intended for clinical use most likely contain not only SP-B but also SP-C. The use of recombinant SP-C (rSP-C) in surfactant preparations is under investigation, to establish the efficiency of this SP-C on the surface tension lowering activities of surfactant[111, 114-116]. With regard to surface tension lowering activity the rSP-C surfactant (Altana, Konstanz, Germany) has similar results to natural surfactant[117, 118]. This rSP-C surfactant contains an SP-C that is an analogue of human SP-C; it contains phenylalanine instead of two cysteines in positions 4 and 5 of the human SP-C sequence, and isoleucine instead of methionine in position 32. However, the effects of these SP-C-analogues on the uptake, or more generally on the metabolism of surfactant, is unknown. Our group has shown that the uptake of surfactant lipids by alveolar type II cells and alveolar macrophages is regulated by SP-C (Poelma et al., submitted), and thus the influence of recombinant SP-C on the surfactant metabolism needs to be clarified.

Additional factors influencing surfactant uptake
Finally, besides factors related directly to surfactant, our group has shown that multiple 'environmental' factors influence and affect the surfactant metabolism. Some of these factors have been described previously; for example, calcium has been shown to influence the metabolism, as in its presence PG promotes association of SP-A and DPPC[119, 120], and was demonstrated to affect the function of SP-B[29, 121]. The effects or influences of these alveolar factors (e.g. divalent cations as suggested by the study of our group with regard to the effects of SP-B and SP-C), are also underlined by the fact that significant differences between *in vivo* and *in vitro* results were demonstrated, even when the absence or dilution of known co-factors such as calcium were compensated for. It should be emphasized that in our opinion the use of *in vitro* experiments is indeed useful, although extrapolation of the results to the *in vivo* situation has to be made carefully. Using a similar technique to conduct studies both *in vivo* and *in vitro* enables the researcher to compare the results and might help to clarify the complex mechanism of the regulation of the uptake of surfactant lipids by alveolar cells *in vivo*.
In addition, although most studies on the uptake of surfactant have focused on healthy animals, many different diseases can disturb the surfactant system, and the presence of cytokines and other inflammatory parameters are known to affect the presence of surface active surfactant in the lung. For example, tumor necrosis factor (TNF)-α, interleukin (IL) -1, interferon (IFN)-γ are known to influence the production of SP-A, SP-B and SP-C, which regulates the uptake of surfactant by alveolar cells, and thus affects the total metabolism[122-126]. In addition, prenatal steroids have been demonstrated to increase surfactant synthesis[62].

Future studies
Because the uptake of surfactant in healthy adult animals has to some extent been clarified, future research could focus on the uptake of surfactant-like liposomes in diseased animals, using different models for diseased animals. Possible irregularities in the uptake and thus the endogenous surfactant metabolism might be elucidated. The known regulatory factors, at least those clarified up to now, will then provide options to restore normal metabolism by influencing the uptake. For example, if uptake of surfactant is reduced in a certain disease state, it might be beneficial to increase the concentration of PG within the surfactant preparation used for therapy. In other words, clarifying regulating factors in the surfactant uptake and uncovering irregularities in the metabolism, or more specifically in the uptake of surfactant,

allows to develop an exogenous surfactant preparation that is disease specific by modifying the composition depending on the underlying deviation from the normal situation.

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CHAPTER 2

In vivo and in vitro uptake of surfactant lipids by alveolar type II cells and macrophages

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Abstract

The uptake of fluorescent-labeled liposomes (with a surfactant-like composition) by alveolar macrophages and alveolar type II cells was studied using flow cytometry: *in vivo* by instillation of the labeled liposomes in the trachea of ventilated rats followed by isolation of the alveolar cells and determination of the cell-associated fluorescence and *in vitro* by incubation of isolated alveolar cells with the fluorescent liposomes. The results show that the uptake of liposomes by the alveolar cells is time- and concentration dependent. *In vivo* alveolar macrophages internalize more than three times as many liposomes as alveolar type II cells, whereas *in vitro* the amount of internalized liposomes by these cells is approximately the same. *In vitro* practically all the cells (70 to 75%) internalize liposomes, whereas *in vivo* only 30% of the alveolar type II cells ingest liposomes versus 70% of the alveolar macrophages. These results indicate that *in vivo* only a small subpopulation of alveolar type II cells is able to internalize surfactant liposomes.

Introduction

Pulmonary surfactant lines the alveolar capillary membrane and plays an essential role in normal lung function. It is a complex of lipids and proteins synthesized by alveolar type II cells and stored in lamellar bodies until it is secreted into the alveolar space[1]. Within the alveolus, surfactant transforms to tubular myelin, which unfolds, and the surfactant lipids are rapidly inserted in the lipid monolayer present at the air-liquid interface[1]. During a breathing cycle, lipids are squeezed out of the monolayer. In order to maintain the primary function of the monolayer, i.e. reduction of the surface tension, the loss of lipids from the monolayer has to be compensated by renewed insertion of lipids. As the *de novo* synthesis of surfactant is insufficient to correct the natural loss (i.e. inactivation of surfactant) the alveolar type II cell not only produces newly synthesized surfactant but also reutilizes inactivated surfactant, derived from the alveolar space.

The recycling of surfactant is a major pathway for surfactant in the alveolar space[2]; clearance by the mucociliar pathway[3] or degradation by alveolar type II cells and alveolar macrophages appears to be less important in the removal of surfactant lipids from the alveolar space.

One important factor in the complex system of surfactant synthesis, secretion, recycling, clearing and degradation, is the uptake of surfactant lipids by alveolar type II cells and alveolar macrophages. Numerous *in vitro* studies have demonstrated that both cell types can internalize surfactant lipids, though their relative contribution in the uptake of surfactant lipids in the lung remains obscure. According to Miles et al., based on *in vitro* studies, alveolar macrophages may be responsible for all the catabolism of surfactant lipids[4].

In contrast, results of *in vivo* experiments suggest a primary role of alveolar type II cells in the uptake of surfactant lipids rather than alveolar macrophages[5, 6], however, a recent study suggests an equal contribution of both alveolar type II cells and alveolar macrophages in the uptake of surfactant lipids *in vivo* in the presence of SP-A[7].

Though differences in methodology may underlie these contradictory findings, another explanation may be the difference between *in vivo* and in *vitro* experiments. Study of the uptake of lipids by alveolar cells *in vivo* and *in vitro* using a similar technique has the advantage that the contribution of the alveolar type II cells and alveolar macrophages in the uptake of lipids in the lung can be assessed, as can the influence of cell isolation and environmental factors. In the present study, we have studied the uptake of fluorescent-labeled liposomes by alveolar type II cells and alveolar macrophages *in vivo* as well as *in vitro* using flow cytometry. In contrast to most studies on the uptake of surfactant lipids by type II cells or alveolar

macrophages which focused on its main component, saturated phosphatidylcholine (PC) (i.e. dipalmitoyl phosphatidylcholine; DPPC)[5, 6, 8], in the present study we used fluorescent liposomes with a composition similar to natural surfactant: DPPC, PC, phosphatidyl glycerol (PG), phosphatidyl inositol (PI), phosphatidyl ethanolamine (PE) and cholesterol (weight ratio 55:21:8:2:6:8)[3].
An advantage of using flow cytometry to study the uptake of lipids by alveolar type II cells and alveolar macrophages is that it allows to determine whether all the cells are able to internalize lipids or only subpopulations. To date, it is not known whether all alveolar macrophages and/or alveolar type II cells are involved in the uptake of lipids. We have developed a new method to study the uptake of surfactant lipids by alveolar type II cells and alveolar macrophages *in vivo* as well as *in vitro* using fluorescent-labeled surfactant-like liposomes.

Materials and methods

Ethical Guidelines
This study was approved by the Institutional Animal Committee at the Erasmus University Rotterdam.

Liposome preparation
To prepare surfactant-like liposomes, the following lipids were mixed; dipalmitoyl phosphatidylcholine (DPPC), phosphatidyl choline (PC), phosphatidylglycerol (PG), phosphatidyl inositol (PI), and rhodamine labeled phosphatidyl ethanolamine (PE)(Rhodamine DHPE; Molecular Probes, Leiden, The Netherlands) and cholesterol in a weight ratio of 55:21:8:2:6:8 and dried under a stream of nitrogen gas. The lipids were purchased from Sigma, Zwijndrecht, The Netherlands, unless stated otherwise. The liposomes were suspended in PBS at a concentration of 1 mg lipids/ml using glass pearls and vortexing. Immediately prior to use, the liposome suspension was sonicated for 2 min on ice using an ultrasonic disintegrator (Branson Sonifier 250, Danbury, USA) to prepare small unilamellar liposomes. These liposomes were used, unless stated otherwise. Large multilamellar liposomes were prepared by sonicating the liposome suspension in a water bath sonicator (Branson 5210, Danbury, USA) at 37°C for 3 min. The size of the liposomes was determined by dynamic light scattering at 25°C with a Malvern 4700 system using a 25 mW He-Ne laser (NEC, Tokyo, Japan) and the automeasure version 3.2 software (Malvern Ltd, UK). As a measure of particle size distribution of the dispersion, the system reports a polydispersity index (p.d.). This index ranges from 0.0 for a monodisperse and up to 1.0 for an entirely polydisperse dispersion.The 'small' liposomes had a size of 161 nm p.d. 0.35 and the 'large liposomes had a size of 1455 nm p.d. 0.65, significantly larger than the 'small' liposomes.

Intratracheal instillation of fluorescent liposomes
The studies were performed with male Sprague-Dawley rats (IFFA Credo, The Netherlands) with a bodyweight of 314 ± 18 g. After induction of anesthesia with a mixture of nitrous oxide (66%), oxygen (33%) and isoflurane (1-2%) a sterile polyethylene catheter (0.8 mm o.d.) was inserted into one of the carotid arteries. The animals were then tracheotomized and a sterile metal cannula was inserted into the trachea.
After these surgical procedures, gaseous anesthesia was ended and replaced with an intraperitoneal injection of pentobarbital sodium (60 mg/ml, Nembutal®, Algin BV, Maassluis, The Netherlands) at a dose of 30 mg/kg bodyweight every hour.
Muscle relaxation was induced and maintained by an hourly intramuscular injection of pancuronium bromide (2 mg/kg, Pavulon; Organon Technika, Boxtel, The Netherlands). The

animals were then mechanically ventilated with a Servo® ventilator 300 (Siemens-Elema, Solna, Sweden) set to pressure control mode using a frequency of 30/min, an inspiratory/expiratory ratio of 1:2, a positive end-expiratory pressure (PEEP) of 2 cm H_2O, a peak inspiratory pressure (PIP) of 12 cm H_2O and FiO_2 was set to 1.
Before instillation of the labeled liposomes, PEEP was increased to 6 cm H_2O and PIP was increased to 26 cm H_2O. After disconnection from the ventilator, the liposomes were administered intratracheally at the indicated doses. The suspension of liposomes (1 mg lipids/ml, unless stated otherwise) was administered as a bolus of 3 ml/kg followed by a bolus of air (12 ml/kg), directly into the endothracheal tube via a syringe, and the animals were immediately reconnected to the ventilator. Thirty min after instillation of the liposomes PEEP was reduced to 2 cm H_2O and PIP to 12 cm H_2O.
Arterial blood gases were measured with conventional methods (ABL 505, Radiometer, Copenhagen, Denmark) at start of ventilation, immediately after instillation of the liposomes and every 30 minutes thereafter. One hour after ventilation (unless stated otherwise) the animals were sacrificed by exsanguination via the abdominal aorta and the alveolar cells were isolated to determine the cell-associated fluorescence. Control animals were sacrificed immediately after anesthesia and their isolated alveolar type II cells and alveolar macrophages were used to correct for auto-fluorescence.

Instillation of a large volume (30 ml/kg) of the liposomes
After the surgical procedures one group of animals was instilled with a large volume of fluorescent-labeled liposomes (30 ml/kg; 1 mg lipids/ml). The liposomes were retrieved from the lungs immediately after instillation (recovery 90%). One hour after instillation, the animals were sacrificed and alveolar type II cells and alveolar macrophages were isolated and cell-associated fluorescence was determined.

Isolation of alveolar type II cells and alveolar macrophages
Prior to isolation of the cells, the thorax was opened and the blood cells were removed from the lungs by perfusing the pulmonary artery with saline (37°C) supplemented with 20 IE heparin (Leo Pharma, Weesp, The Netherlands). The lungs were removed from the thoracic cavity en bloc and lavaged with 10 ml of solution 1 [140 mM NaCl, 5 mM KCl, 2.5 mM $Na_2HPO_4.2H_2O$, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethaesulfonic acid), 6 mM glucose, 0.2 mM EGTA (ethylene glycol-bis- (β-amino ethyl ether) N,N'-tetra-acetic acid), pH 7.40] at 22°C. This procedure was repeated four times. The lung lavages were pooled per animal and centrifuged (100x *g*; 10 min; 4°C). The cellular pellet, i.e. alveolar macrophages, were suspended in solution 2 (140 mM NaCl, 5 mM KCl, 2.5 mM phosphate buffer, 10 mM HEPES, 6 mM glucose, 2.0 mM $CaCl_2$ and 1.3 mM $MgSO_4$) to a concentration of $2x10^6$ cells/ml and stored on ice until further use. The alveolar type II cells were isolated according to Dobbs et al.[9]. Alveolar type II cells were suspended in solution 2 at a concentration of $2x10^6$ cells/ml and stored on ice until further use. Alveolar macrophages were identified using monoclonal antibodies specific for rat macrophages (ED9) and alveolar type II cells were identified using an alkaline phosphatase assay as described by Edelson et al.[10]. The average yield of alveolar type II cells was $16x10^6$ and $5x10^6$ alveolar macrophages per rat.

In vitro assay to determine the uptake of fluorescent-labeled liposomes
Alveolar type II cells and alveolar macrophages were isolated from control animals as described above and were suspended in solution 2 to a concentration of $2x10^6$ cells/ml. A total of $3x10^5$ cells were incubated with various concentrations of liposomes at 37°C (final volume 500 µl) in a shaking water bath. After 1 hour, the incubation was terminated by addition of 2 ml of ice-cold PBS. The cell suspension was centrifuged at 100x *g* for 10 min at 4°C. The super-

natant was removed and the cells were suspended in 2 ml of ice-cold PBS and centrifuged again. This wash procedure was repeated twice. Finally, the pellet was resuspended in 200 µl cold PBS and cell-associated fluorescence was determined as described below.

Flow cytometry
Cell-associated fluorescence of the alveolar type II cells and alveolar macrophages as a measure for internalized liposomes was determined using flow cytometry (FACSCalibur; Becton Dickinson; Mountain View, USA). The cell-associated fluorescence of 15,000 cells was determined. Alveolar macrophages and alveolar type II cells derived from control animals were used in each experiment to determine the autofluorescence of the cells. Subsequently, the mean cell-associated fluorescence was determined only for those cells that had a higher fluorescence than that caused by auto-fluorescence (gated cells). For the cell sorting experiment, the FACSCalibur was set to the sorting mode, and only those cells with a mean fluorescence higher than the autofluorescence were collected and analyzed.

Localization of cell-associated fluorescence
To inspect the localization of the cell-associated fluorescence confocal micrographs of alveolar cells were obtained using a confocal microscope (Zeiss LSM 410, Jena Germany). Images were created with standard objectives and photomultiplier tubes dedicated to the appropriate excitation and emission spectra of rhodamine (excitation 541 nm and emission 572 nm). Images of alveolar cells were serial sectioned at a depth of 0.5 µm, to distinguish cell-membrane associated fluorescence from true intra cellular fluorescence.

Statistical analysis
Differences in blood gas values over time were analysed using a repeated measurement ANOVA followed by an independent *t*-test. Differences between animals that received fluorescent-labeled liposomes and the control group were determined using an independent samples *t*-test. Differences between the animals receiving a normal volume of liposomes and the animals receiving a high volume of liposomes were analyzed using an *independent t-test.* Differences were considered statistically significant at a $p < 0.05$. Values are expressed as mean ± SEM

Results

Isolated alveolar type II cells and alveolar macrophages.
The forward and sideward scatter plots in Fig. 1 demonstrate that alveolar type II cells and alveolar macrophages isolated from controls consist of heterogeneous cell populations. The cell-associated fluorescence of alveolar cells isolated from rats one 1 hour after intratracheal instillation of fluorescent-labeled liposomes, was clearly higher than the control cells. The cell-associated fluorescence of the alveolar macrophages is higher than the fluorescence of the alveolar type II cells, suggesting more uptake of fluorescent liposomes by alveolar macrophages. Instillation of the fluorescent-labeled liposomes had no significant effect on oxygenation levels of the animals during ventilation (results not shown).

In vivo uptake of fluorescent liposomes by alveolar cells
To characterize the in vivo uptake of fluorescent-labeled liposomes by alveolar cells, the cells were isolated at different time points (0.5 to 3 hours) after instillation of the fluorescent-labeled liposomes. The cell-associated fluorescence of alveolar type II cells and of alveolar macrophages demonstrated a time-dependent increase reaching a plateau in 1 hour (Fig. 2). In

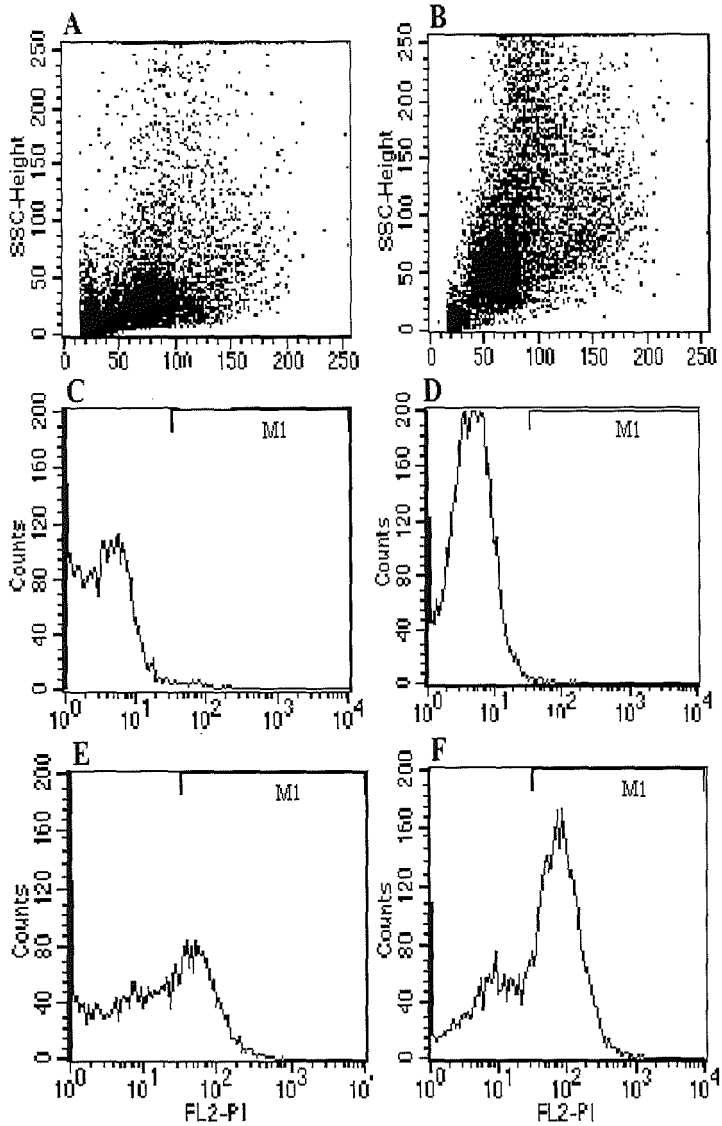

Fig 1. *Flow cytometry of alveolar cells*
Alveolar type II cells (A,C,E) and alveolar macrophages (B,D,F) were isolated from control animals (A-D) and from animals one hour after intratracheal instillation of fluorescent liposomes (E,F). The forward and sideward scatter plots demonstrate the presence of heterogeneous populations of alveolar type II cells (A) and alveolar macrophages (B). Autofluorescence of control alveolar type II cells (C) and control alveolar macrophages (D) was determined. Total cell-associated fluorescence was determined of alveolar type II cells (E) and alveolar macrophages (F) isolated from animals that were intra tracheally instilled with fluorescent liposomes. Marker M1 (C-F) indicates the mean cell-associated fluorescence of the cells that have internalized the fluorescent liposomes (gated cells).

addition, concentration dependency was determined by instilling fluorescent-labeled liposomes of different concentrations (0.5, 1, 2, 3, 5 mg/ml) and isolating the alveolar cells 1 hour after instillation (Fig. 3). At a concentration of 1 mg/ml the cell-associated fluorescence of the alveolar type II cells was half maximal whereas for alveolar macrophages the half maximal uptake was observed at an intratracheal dose of 2 mg/ml fluorescent liposomes.

The percentage alveolar cells involved in the uptake of fluorescent liposomes (gated cells) reached a maximum at a concentration of 1 mg/ml (Fig. 3). Approximately 30% of the alveolar type II cells participated in the uptake of fluorescent-labeled liposomes. In contrast, about 70% of the alveolar macrophages internalize the fluorescent-labeled liposomes (Fig. 3). At an intratracheal dose of 3 mg fluorescent liposomes, the uptake by the alveolar cells was maximal. The mean cell-associated fluorescence of the alveolar macrophages involved in the uptake was 3.26 ± 1.24 times higher than that of the alveolar type II cells; this indicates 3.26 times more uptake of liposomes by alveolar macrophages than alveolar type II cells.

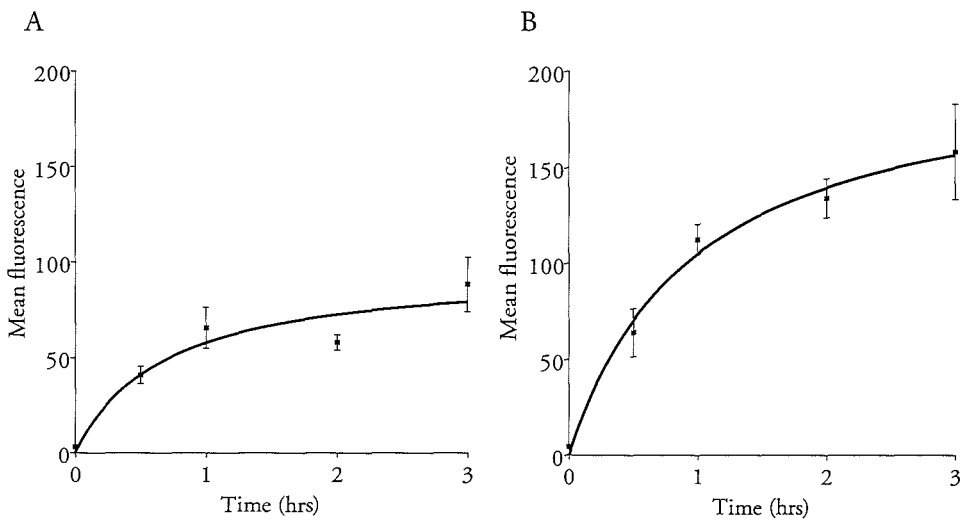

Fig 2. *Time-dependent uptake of fluorescent liposomes*
At every time point (30 min, 1, 2 and 3 hours after instillation of the liposomes), 5 animals were sacrificed, alveolar type II cells (A) and macrophages (B) isolated, and mean cell-associated fluorescence was determined. At all time points fluorescence differed significantly from the control group (values are mean ± SEM).

Uptake of unilamellar vs. multilamellar liposomes
As demonstrated by Griese et al.[11], smaller sized particles are preferentially taken up by the alveolar type II cells. We studied the effect of the size of the labeled liposomes on the uptake *in vivo* by instilling either small unilamellar liposomes, created by ultrasonification, or large multilamellar liposomes, sonicated in a warm water bath sonicator[12]. No differences were observed in the uptake of both types of liposomes, unilamellar or multilamellar (Table 1).

Localisation of the cell-associated fluorescence
Confocal laser microscopy was used to ascertain that the measured cell-associated fluorescence was caused by intracellular presence of fluorescent labeled liposomes. Confocal scans through

the middle of the cell (Fig. 4) show that the fluorescence is not limited to the circumference of the cell and shows a punctuate fluorescence throughout the cell except for the nucleus.

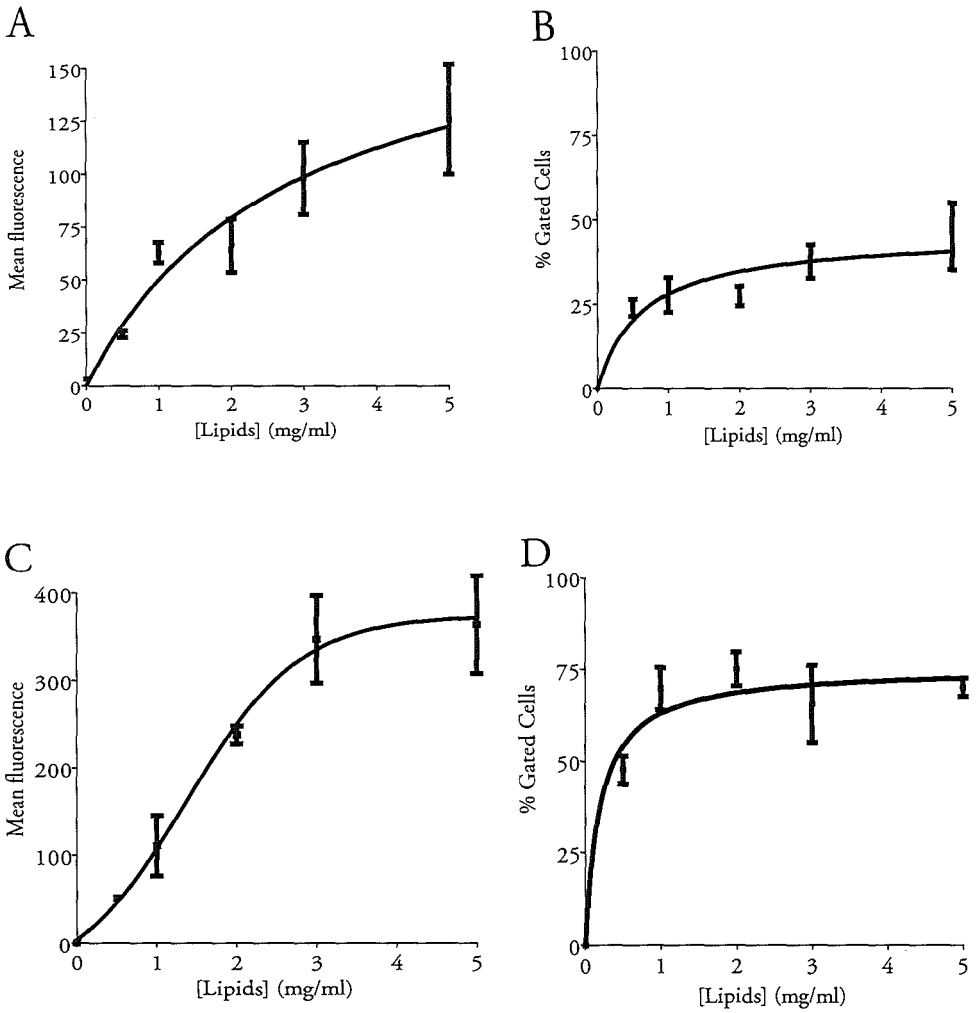

Fig 3. *Concentration-dependent uptake of liposomes in vivo*
One hour after instillation of the indicated concentrations of fluorescent liposomes, the alveolar cells were isolated. The mean cell-associated fluorescence of the gated alveolar type II cells (A) and alveolar macrophages (C) was determined. In addition, the percentage gated alveolar type II cells (B) and alveolar macrophages (D) was determined. At all concentrations fluorescence and percentage gated cells differed significantly from the control groups (n=4 rats at every concentration; values are mean ± SEM).

Identification of cells that internalize the fluorescent liposomes
We found that in vivo only 30% of the type II cells isolated from rats intratracheally instilled with fluorescent liposomes were involved in the uptake of fluorescent liposomes. The average percentage of cells identified as alveolar type II cells in the alveolar type II cell isolate was

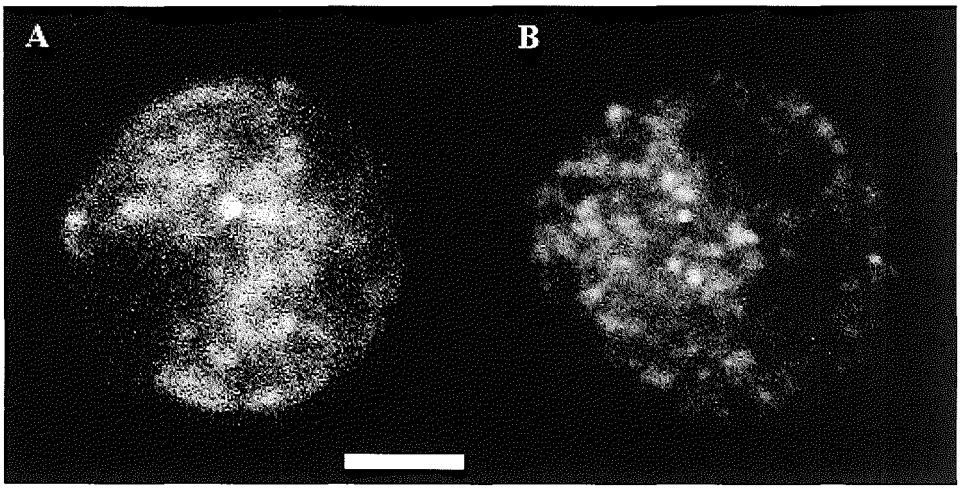

Fig 4. *Confocal microscopic images of alveolar type II cells and alveolar macrophages*
Alveolar type II cells (A) and alveolar macrophages (B) isolated one hour after instillation of fluorescent-labeled liposomes were mounted on glass cover slips. Confocal laser microscopy demonstrated a punctuate distribution of fluorescent label not limited to the cell surface, which did not localize to the nuclei, indicating internalization of the fluorescent liposomes by both cell types. Since untreated control cells did not show this fluorescence at the same microscope settings, we conclude that the observed internal fluorescence of treated cells was due to the uptake of fluorescently labeled liposomes. Bar indicates 5 μm.

approximately 80%. Theoretically, it is possible that a substantial number of the cells that internalize fluorescent liposomes in alveolar type II cell isolate are not type II cells but contaminating cells such as macrophages, etc. To exclude this possibility, rats were intratracheally instilled with fluorescent liposomes. The alveolar type II cells and alveolar macro-

Table 1. *Size-dependent uptake of fluorescent liposomes*

	Alveolar type II cells		Alveolar macrophages	
	Mean fluorescence	Gated cells	Mean fluorescence	Gated cells
Control	2 ± 1	2 ± 1	4 ± 1	1 ± 1
Small liposomes	66 ± 8	28 ± 4	112 ± 17	70 ± 15
Large liposomes	44 ± 10	16 ± 7	117 ± 36	52 ± 5

Alveolar cells were isolated one hour after instillation of the indicated fluorescent liposomes. Small liposomes: unilamellar fluorescent liposomes; large liposomes: multilamellar fluorescent liposomes. Mean fluorescence: mean cell-associated fluorescence; gated cells: percentage gated cells (n = 3 rats; values are mean ± SEM).

Table 2. *Identification of fluorescent cells*

	Type II cells isolate	Macrophage isolate
% type II cell	80 ± 5	N.D.
% Macrophage	1.7 ± 2.1	92 ± 5

Alveolar cells were isolated one hour after instillation of fluorescent liposomes; fluorescent cells were collected and identified. N.D.: not detected (n = 3 rats, values are mean ± SEM).

phages were isolated. The alveolar cells with a mean fluorescence higher than the autofluorescence of the control cells were sorted and collected. As shown in Table 2, 80 ± 5% (n=3) of these fluorescent cells present in the alveolar type II cell isolate were identified as alveolar type II cells using alkaline phosphatase assay, whereas 92 ± 5% (n=3) of the alveolar macrophages could be identified as macrophages as was demonstrated using a monoclonal antiserum directed against macrophages (ED9).

Distribution of the liposomes

To determine whether the low number of alveolar type II cells internalizing the liposomes was due to an inhomogeneous distribution of liposomes in the lungs, one group of animals was instilled with a high volume of fluorescent-labeled liposomes.

Instillation of a large volume of liposomes which was immediately redrawn from the lungs, did not result in a significant difference in the percentage of cells responsible for the fluorescence, nor was there any significant difference in the level of fluorescence between the animals receiving the liposomes as a bolus and the animals receiving a high volume of liposomes (Table 3). Interestingly, the number of alveolar macrophages involved in the uptake of liposomes was significantly lower in the high volume group than in the low volume, standardly used, group.

Table 3. *Distribution of fluorescent liposomes*

	Alveolar type II cells		Alveolar macrophages	
	Mean fluorescence	Gated cells	Mean fluorescence	Gated cells
Control	2 ± 1	2 ± 1	4 ± 1	1 ± 1
Low volume	66 ± 8	28 ± 4	112 ± 17	70 ± 15
Large volume	62 ± 7	18 ± 6	123 ± 19	38 ± 5*

Alveolar cells were isolated one hour after instillation of fluorescent liposomes with the indicated method. Low volume: 3 ml/kg liposomes followed by bolus of air; large volume: 30 ml/kg liposomes immediately withdrawn from the lungs (recovery 90%). Mean fluorescence: mean cell-associated fluorescence; gated cells: percentage gated cells (n = 3 rats; values are mean ± SEM).

* Significantly smaller than low volume value, $p < 0.05$ (by unpaired t-test).

In vitro measurement of uptake

In vitro experiments with different concentrations of fluorescent-labeled liposomes demon-

strated an apparent maximal uptake at 20 µg/ml for both cell types (Fig. 5). The percentage alveolar type II cells responsible for the uptake was maximal at 100 µg/ml at 67.6 ± 11.1%. The percentage alveolar macrophages reaches its maximum of 73 ± 34.7% at 20 µg/ml (Fig. 5). The mean cell-associated fluorescence did not significantly differ between the alveolar type II cells and the alveolar macrophages.

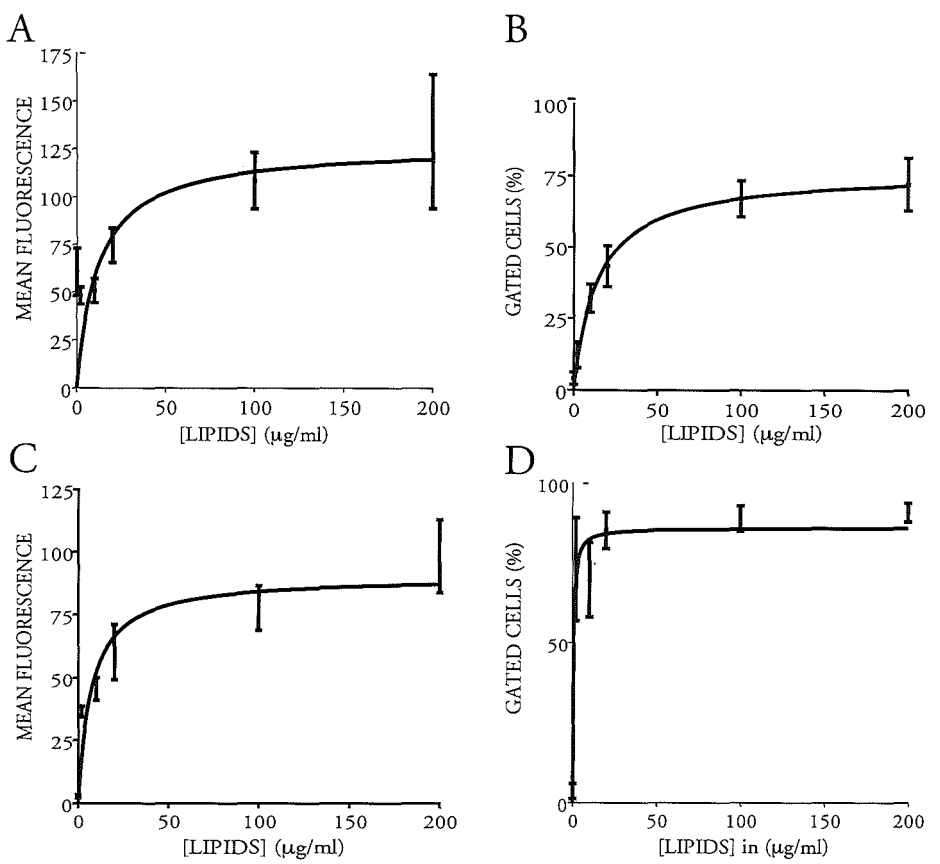

Fig 5. *Concentration-dependent uptake of liposomes in vitro*
Alveolar cells were isolated from control animals and incubated for 1 hour at 37°C with the indicated concentrations of fluorescent liposomes. The mean cell-associated fluorescence of gated alveolar type II cells (A) and alveolar macrophages (C) was determined. In addition, the percentage gated alveolar type II cells (B) and alveolar macrophages (D) was determined. At all concentrations fluorescence and percentage gated cells differed significantly from the control group. (n= 4 incubations at every concentration; values are mean ± SEM).

The mean fluorescence of the alveolar macrophages *in vitro* is 5.12 ± 1.01 times lower in comparison to the fluorescence of the alveolar macrophages *in vivo*.
In order to get an impression of the amount of lipids that are only bound to the cellular membranes of the cells, we have incubated the cells on ice with the fluorescent liposomes for 1 hour. For alveolar type II cells, 12 ± 4% (n=3) of the total cell population had a mean

fluorescence [28 ± 7 (n=3)] higher than the autofluorescence, for alveolar macrophages 18 ± 3% (n=3) of the cells had a mean fluorescence [36 ± 5 (n=3)] higher than the autofluorescence.

Discussion

In the present study we used fluorescent-labeled liposomes to study the uptake of lipids by alveolar cells. For the *in vivo* experiments, these liposomes were intratracheally instilled in mechanically ventilated rats. The choice to mechanically ventilate the animals was made on the basis of ensuring that the liposomes are instilled in a completely opened lung, rather than in a lung in which atelectesis are present. The used ventilator settings, peak of 26 cm H_2O and a PEEP of 6 cm H_2O, resulted in a mean airway pressure of 13 cm H_2O. It has been demonstrated that ventilating with mean airway pressure up to 15 cm H_2O does not affect the surfactant metabolism, and does not induce lung injury[13].

By using different concentrations we have demonstrated a concentration-dependent uptake, reaching a maximum at 3 mg/ml. A time-dependent uptake was demonstrated by isolating the alveolar cells at different time points after instillation of the liposomes, similar to previous studies using radioactive-labeled liposomes[5, 14, 15]. Using a composition similar to natural surfactant diminishes the effects of single components and allows to study the uptake of surfactant as a whole complex of different lipids.

The use of confocal laser microscopy allows to demonstrate the intracellular presence of the fluorescent-labeled liposomes. The cell-associated fluorescence was therefore caused by internalized liposomes rather than by liposomes associated with the cell membrane of the alveolar cells.

The use of fluorescence enabled to quantitate the fluorescence per individual cell, which leads to intriguing results. Interestingly, only 30% of the alveolar type II cells is involved in the uptake of liposomes. After instillation of liposomes with different concentrations, the number of cells taking part in the uptake of the liposomes does not increase above ± 30%. Identification of the isolated cells demonstrated a similar amount of type II cells in the type II cell isolate as reported in other studies[5, 6, 16]. The cells with a fluorescence higher than the autofluorescence were identified as type II cells, demonstrating that the measured cell-associated fluorescence was indeed caused by type II cells. The low number of alveolar type II cells involved in the uptake of lipids *in vivo* is intriguing, as the *in vitro* experiments demonstrated a much larger number of type II cells (70%) participating in the uptake. Instillation of a high volume of labeled liposomes, distributing the liposomes throughout the lung, did not result in an increase in type II cells taking up the liposomes. Hence, the low number of participating type II cells *in vivo* is not caused by an insufficient distribution or by cells other than alveolar type II cells in the cell isolate. In other words, the difference is either caused by the isolation procedure, or is due to the absence of environmental influences *in vitro*. It seems unlikely that the isolation procedure stimulates the alveolar type II cells to take up lipids. It has been observed that a stressful isolation procedure can cause secretion of lamellar bodies[17]; in addition, the turnover, degradation and resecretion continues during the 1 hour incubation. Therefore, the measured fluorescence in vivo is an underestimation of the actual uptake of the fluorescent labeled liposomes. On the other hand, the fluorescence per cell is only slightly increased in vitro, whereas the number of cells participating in the uptake is significantly increased. Therefore, these results suggest a possible regulating effect of the alveolar environment on the uptake of lipids by alveolar type II cells, as well as the presence of a subpopulation of alveolar type II cells which are activated either during the isolation procedure or by the absence of the environmental factors, or degredate, c.q. resecrete the fluorescent labeled liposomes more rapidly. Alveolar type II cells have been shown to interact with alveolar

macrophages[18, 20], although it remains unclear if these cells regulate each others uptake of lipids. In addition, an important role of the environment is also seen in the uptake of lipids by alveolar macrophages. Both *in vivo* and *in vitro*, the same number of alveolar macrophages takes part in the uptake (70%), although most interestingly the alveolar macrophages take up more lipids *in vivo* than *in vitro*. The instillation of liposomes with different concentrations demonstrated that the uptake of liposomes by alveolar macrophages reaches a plateau phase, both *in vivo* and *in vitro*. When comparing the mean fluorescence per cell at this plateau it is obvious that *in vivo* the alveolar macrophages take up approximately 5 times as many liposomes than *in vitro*. In other words, both the alveolar type II cell and alveolar macrophage interact with the environment.

Additionally the results of the instillation of a high volume of liposomes suggest the presence of subpopulations within the alveolar macrophages. When a high volume of liposomes (30 ml/kg) is instilled and removed from the lung, a number of alveolar macrophages is removed as in a lung lavage. One hour after instillation and withdrawal of the high volume the alveolar cells were isolated and cell-associated fluorescence was determined. The alveolar type II cells showed no significant difference compared with the cells derived from animals instilled with the normal volume (1 ml). In contrast, the alveolar macrophages showed a significant decrease in the number of cells taking up the liposomes. The mean fluorescence per cell did not differ significantly, suggesting that a part of the cells that are involved in the uptake of fluorescent liposomes were removed by the instillation and withdrawal of a high volume of liposomes. In this primary 'lavage' the most mobile cells are removed, whereas the more resident cells are not likely to be removed. Although there might be influx of new alveolar macrophages by chemotaxis, their contribution in the clearance of the liposomes from the alveolar space is time-dependent as demonstrated in the present study; we therefore conclude that the relatively short time of exposure to the liposomes of recently influxed cells can explain the minor contribution of these newly influxed cells. The mean fluorescence per cell did not differ, indicating that the cells were not negatively influenced by this lavage, although the low number of cells involved suggest that the liposomes are preferentially taken up by the mobile macrophages rather than by the resident, adherent cells.

To date, their relative contribution is under discussion, some studies indicate a minor role of the alveolar macrophages in the uptake of lipids[5], others indicate a larger contribution of these cells to the clearance of surfactant[4, 7]. We have found comparing the alveolar type II cells with the alveolar macrophages, that the alveolar macrophages take up approximately 2-3 times more liposomes. When correcting for the number of cells within the lung, it is clear that the alveolar type II cells contribute ± 60-70% of the clearance of surfactant from the alveolar space. There are about 10-15 times as many type II cells than macrophages (calculations using ref.[21]), although only 30% of the type II cells are involved in the clearance of the liposomes compared to 70% of the alveolar macrophages, the alveolar type II cells still outnumber macrophages 5-7 times with regard to the number of cells involved in the clearance of liposomes. The macrophages take up 2-3 times more liposomes than the alveolar type II cells. Therefore, the alveolar type II cells contribute about 2-3 times more than the alveolar macrophages.

In the present study we used fluorescent-labeled liposomes with a composition similar to that of natural surfactant, without surfactant proteins. Although studies in the past have demonstrated a possible role of the apoproteins, especially SP-A, on the uptake of surfactant by both alveolar type II cells and alveolar macrophages[7], the present study focuses on the method on measuring the uptake rather than studying the role of the apoproteins. The absence of the surfactant proteins might account for the different findings in the contribution of alveolar macrophages in the present study, although the inter-species variation is another option.

In summary, using fluorescent-labeled liposomes, we have demonstrated an important role of the environment on the uptake of surfactant liposomes by alveolar cells, as well as the presence

of possible subpopulations alveolar type II cells and macrophages, that are involved in the uptake.

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CHAPTER 3

A common pathway for the uptake of surfactant lipids by alveolar cells

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Abstract

The uptake of different surfactant lipids: dipalmitoylphosphatidylcholine (DPPC), phosphatidylglycerol (PG) or phosphatidylinositol (PI) and liposomes with a surfactant-like composition by alveolar type II cells (alveolar type II cells) and macrophages (alveolar macrophages) was studied in vitro.
Fluorescent labeled liposomes containing either 86% of the studied lipid i.e. DPPC, PG, PI and 6% labeled phosphatidylethanolamine (PE) and 8% cholesterol or a lipid mixture similar to surfactant (DPPC, PG, PI, phosphatidylcholine, PE and cholesterol in a weight ratio of 55:8:2:21:8:6) were incubated with alveolar macrophages and alveolar type II cells. The cell-associated fluorescence assessed by flow cytometry, demonstrated a higher uptake of PG and PI by both alveolar macrophages and alveolar type II cells, and a lower uptake of DPPC by alveolar macrophages. In addition, less alveolar type II cells take up DPPC whereas there are no differences for the alveolar macrophages in the number of cells involved in the uptake.
Competition experiments with Texas-Red labeled liposomes and either DPPC liposomes or PI liposomes labeled with Bodipy indicated that all these liposomes are internalized via the same pathway by alveolar cells. Thus, lipid composition directly influences the (re)uptake of surfactant.

Introduction

The alveolus is lined with a thin layer of lipids and proteins, called surfactant and this surface active agent has an essential role in maintaining normal lung function. Pulmonary surfactant is produced by alveolar type II cells. By weight, approximately 90% of surfactant consists of lipids. Although, the lipid composition varies in different species, its major component is phosphatidylcholine (PC; 70-80%) of which nearly 50% is saturated dipalmitoylphosphatidylcholine (DPPC). DPPC is the major surface tension-reducing component of surfactant. In addition, surfactant contains variable amounts of phosphatidylglycerol (PG; 7-18%), phosphatitylinositol (PI; 2-4%) and phosphatidylethanolamine (PE; 2-3%)[1]. The remaining percentage consists of other phospholipids and cholesterol[1].
The presence of surfactant within the alveolus is the result of a complex system of production, secretion, uptake and recycling. The production of newly synthesized surfactant has been suggested to be relatively slow, especially in newborn animals[2, 3]. Therefore, inactivated surfactant is reutilized, i.e. recycled. It has been demonstrated that 50-90% of the PC is recycled depending on age and species, where with an increasing age the contribution of recycling decreases[4]. Inactivated surfactant is cleared from the alveolar space mainly by alveolar type II cells[5]. This uptake of surfactant by alveolar type II cells is essential to enable recycling of surfactant. Therefore uptake or reuptake of surfactant plays an important role in the homeostasis of the surfactant metabolism.
It has been shown that there are essential differences in the composition of surfactant between neonates and adults regarding the phospholipids which, combined with the fact that neonates rely more on recycling, suggests an effect of the composition on the uptake of surfactant. Previous studies have demonstrated an effect of surfactant proteins, especially SP-A, on the uptake by alveolar cells[6-9]. However, little is known on the effect of lipid composition on the clearance of surfactant from the alveolar space by alveolar cells, since most studies have focused on internalization of DPPC by both alveolar type II cells and alveolar macrophages. Quintero et al. have shown a more rapid clearance of PG by alveolar macrophages in contrast to DPPC[10], indicating an effect of the lipid composition on the uptake. It remains unclear, however, whether the uptake of surfactant by alveolar type II cells is also affected by lipid compo-

sition. Therefore, we studied the uptake of the major surfactant lipids by alveolar type II cells as well as alveolar macrophages using fluorescent labeled liposomes[11]. In addition, we examined how the different lipids influence each other's uptake by alveolar cells.

Material & methods

Ethical Guidelines

This study was approved by the Institutional Animal Committee at the Erasmus University Rotterdam and complied with NIH guidelines. A total of 30 male Sprague Dawley rats (IFFA Credo, The Netherlands) with an average weight of 280 ± 41 g were used.

Materials

Dipalmitoylphosphatidylcholine (DPPC), egg phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and cholesterol were purchased from Sigma (Zwijndrecht, The Netherlands). PE labeled with Bodipy® and PE labeled with Texas Red® were obtained from Molecular Probes (Leiden, The Netherlands). Both labeled PE lipids are labeled in the head-group.

Liposome preparation

To prepare liposomes, the indicated concentrations of the different lipids were mixed. The mixture of lipids was dried under a stream of nitrogen gas. The liposomes were suspended in PBS at a concentration of 0.5 mg lipids/ml using glass pearls and vortexing. Immediately prior to use, the liposome suspension was sonicated for 2 min on ice using an ultrasonic disintegrator (Branson Sonifier 250, Danbury, USA) to prepare small unilamellar liposomes[11]. The size of the liposomes was determined by dynamic light scattering at 25°C with a Malvern 4700 system using a 25 mW He-Ne laser (NEC, Tokyo, Japan) and the automeasure version 3.2 software (Malvern Ltd, UK). As a measure of particle size distribution of the dispersion, the system reports a polydispersity index (p.d.). This index ranges from 0.0 for a monodisperse and up to 1.0 for an entirely polydisperse dispersion. After ultrasonification the liposomes size ranged from 140 to 165 nm and the polydispersity indexe ranged from 0.2 to 0.35.

Isolation of alveolar cells

Prior to isolation of the cells, the thorax was opened and the blood cells were removed from the lungs by perfusing the pulmonary artery with saline (37°C) supplemented with 20 IE heparin (Leo Pharma, Weesp, The Netherlands). The lungs were removed from the thoracic cavity en bloc and lavaged with 10 ml of solution 1 [140 mM NaCl, 5 mM KCl, 2.5 mM $Na_2HPO_4.2H_2O$, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethaesulfonic acid), 6 mM glucose, 0.2 mM EGTA [ethylene glycol-bis- (β-amino ethyl ether) N,N'-tetra-acetic acid), pH 7.40] at 22°C. This procedure was repeated four times. The lung lavages were pooled per animal and centrifuged (100x *g*; 10 min; 4°C). The cellular pellet, i.e. alveolar macrophages, were suspended in solution 2 (140 mM NaCl, 5 mM KCl, 2.5 mM phosphate buffer, 10 mM HEPES, 6 mM glucose, 2.0 mM $CaCl_2$ and 1.3 mM $MgSO_4$) to a concentration of $2x10^6$ cells/ml and stored on ice until further use. The alveolar type II cells were isolated according to Dobbs et al.[12]. Alveolar type II cells were suspended in solution 2 at a concentration of $2x10^6$ cells/ml and stored on ice until further use. Alveolar macrophages were identified using monoclonal antibodies specific for rat macrophages (ED9) and alveolar type II cells were identified using an alkaline phosphatase assay as described by Edelson et al.[13]. The average yield per rat of alveolar type II cells was $16x10^6$ and $5x10^6$ alveolar macrophages. The purity of the alveolar type II cells was determined and 80 ± 5% of the cells in the type II cell isolate were

identified as alveolar type II cells. The alveolar macrophage isolate had a purity of 92 ± 2% alveolar macrophages as determined by ED9 antibodies.

Uptake of liposomes by alveolar cells

Alveolar type II cells and alveolar macrophages were isolated as described above and were suspended in solution 2 to a concentration of $2x10^6$ cells/ml. A total of $3x10^5$ cells were incubated with various concentrations of the different liposomes at 37°C (unless stated otherwise) (final volume 500 µl) in a shaking water bath. After 1 hour, the incubation was terminated by addition of 2 ml of ice-cold PBS[11]. The cell suspension was centrifuged at 100x *g* for 10 min at 4°C. The supernatant was removed and the cells were suspended in 2 ml of ice-cold PBS and centrifuged again. This wash procedure was repeated twice. Finally, the pellet was resuspended in 200 µl cold PBS and cell-associated fluorescence was determined as described below.

Flow cytometry

As a measure for the amount of internalized liposomes, cell-associated fluorescence of a total of 15,000 alveolar type II cells or alveolar macrophages was determined using flow cytometry (FACSCalibur; Becton Dickinson; Mountain View, USA). The Bodipy® fluorescence was determined in the FL 1 channel whereas the Texas Red® fluorescence was determined in the FL 4 channel. The spill-over of Bodipy in the FL 4 channel as well as Texas Red in the FL 1 channel, was less than 1% and was not corrected for in the double labeling experiments. Control alveolar macrophages and alveolar type II cells were used in each experiment to determine the autofluorescence of the cells. Subsequently, the mean cell-associated fluorescence was determined only for those cells that had a higher fluorescence than that caused by autofluorescence (gated cells)[11]. Cells with a higher fluorescence than that caused by auto-fluorescence were collected and identified using the methods described above, showing similar results for all types of liposomes, 81 ± 6% of the cells were type II cells within the type II cell isolate and 93 ± 5% were alveolar macrophages in the alveolar macrophage isolate.

Localization of cell-associated fluorescence

Confocal laser miscoscopy was performed using a Zeiss LSM 410, with standard objectives and photomultiplier tubes dedicated to the appropriate excitation and emission spectra of the used fluorescent label (Bodipy excitation 503 nm emission 512 nm, Texas Red excitation 589 nm emission 615 nm). Images were serial sectioned at a depth of 0.5 µm to distinguish cell-membrane associated fluorescence from true intracellular fluorescence.

Statistical analysis

The differences in uptake of different components were analyzed using an ANOVA followed by a Bonferroni post hoc test.
Differences were considered statistically significant at a $p < 0.05$. Values are expressed as mean ± SEM.

Results

Concentration dependent uptake of liposomes

To study the uptake of the phospholipid components of surfactant, DPPC, PC, PG and PI, isolated alveolar cells were incubated for 1 hour with liposomes of different compositions. The mean cell-associated fluorescence, as a measurement for uptake of liposomes, of the alveolar type II cells and alveolar macrophages demonstrates a concentration-dependent increase. With

exception of the uptake of PI liposomes by alveolar macrophages and the uptake of PG liposomes by alveolar type II cells, the uptake of all individual liposomes reaches an apparent maximum at 25 μg/ml (Fig. 1A, B). However, the maximum cell-associated fluorescence, e.g. maximal uptake, at the plateau differs between the various liposome compositions.
The 86% PG and 86% PI liposomes are internalized significantly more by alveolar type II cells than normal liposomes (PC, DPPC, PG, PI, PE and cholesterol in weight ratio of 21:55:8:2:6:8). The uptake of 86% PC liposomes and 86% DPPC liposomes does not differ from the normal liposomes, except at a higher lipid concentration of 50 μg/ml, more 86% PC liposomes are internalized (Fig 1A).

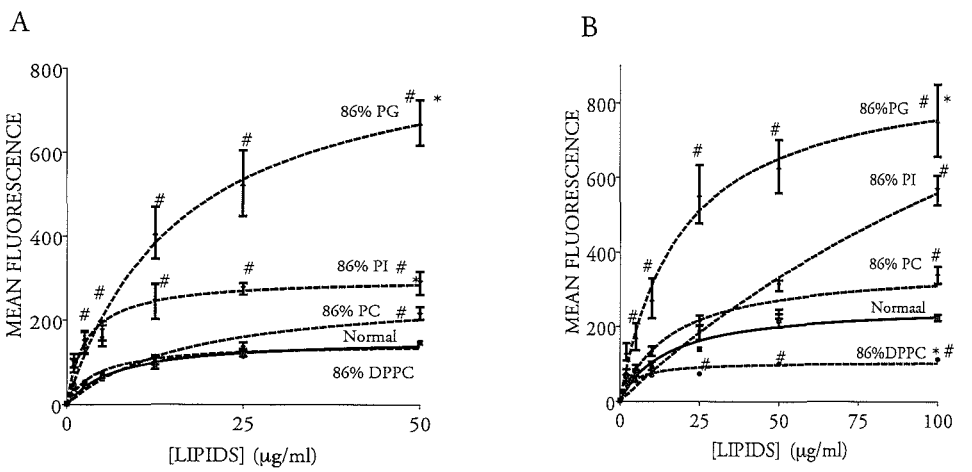

Figure 1. *Concentration-dependent uptake of liposomes*
Alveolar type II cells (A) and macrophages (B) were isolated and incubated for 1 hour at 37°C with the indicated concentrations of either 86% PG (PG:Chol: PE-Bodipy; weight ratio: 86:8:6) or 86% PI (PI:Chol:PE-Bodipy; 86:8:6) or 86% DPPC (DPPC:Chol:PE-Bodipy; 86:8:6) or 86% PC(PC: Chol: PE-Bodipy; 86:8:6) or normal (DPPC:PC:PG:PI:Chol:PE-Bodipy; 55:21:8:2:8:6) liposomes. The mean cell-associated fluorescence was determined of those cells with a higher fluorescence than the auto-fluorescence (n = 4 incubations of $3x10^5$ cells each at every concentration; values expressed as mean ± SEM). # indicates a significant difference compared to the normal liposomes.

There is a significant difference in the uptake of 86% PG liposomes, 86% PI, 86% PC, and normal liposomes by alveolar macrophages at a concentration of 50 μg/ml. The internalization of 86% PG liposomes is significantly higher than that of normal liposomes whereas the uptake of 86% DPPC liposomes by alveolar macrophages is significantly lower than normal liposomes. These results may indicate that uptake of liposomes by alveolar macrophages and alveolar type II is effected by the charge of the liposomes (i.e. 86% PG and 86% PI liposomes are more negatively charged).

Percentage alveolar type II cells and alveolar macrophages involved in the uptake
The percentage gated alveolar type II cells and alveolar macrophages involved in the uptake of liposomes also demonstrate a concentration-dependent increase, reaching a plateau for all groups of liposomes (Fig. 2 A, B). The percentage alveolar macrophages involved in the uptake

of liposomes is not significantly affected by the charge of the liposomes (Fig. 2B). In contrast, the number of alveolar type II cells internalizing liposomes is affected by the charge of the liposomes; although only the 86% PI liposomes are capable of significantly increasing the number of alveolar type II cells taking up the liposomes. More interesting, however, is the significantly lower percentage of alveolar type II cells taking part in the uptake of 86% DPPC liposomes; 29 ±4.5% versus 72.7 ± 2.3% involved in the internalization of normal liposomes at a concentration of 50 µg/ml (Fig. 2A).

Localization of cell-associated fluorescence

To ascertain that the cell-associated fluorescence was located within the cell rather than aspecific binding to the outer membrane of the cell, confocal laser microscopy was used. The confocal scans through the middle of the cell show a punctuate fluorescence throughout the cell limited to its circumference with the exception of its nucleus. Both labels, the TexasRed-labeled PG liposomes (Fig. 3 A,C) and Bodipy-labeled DPPC liposomes (Fig. 3 B, D) were internalized by the alveolar type II cells and alveolar macrophages as untreated cells did not show any fluorescence at the same microscopic settings.

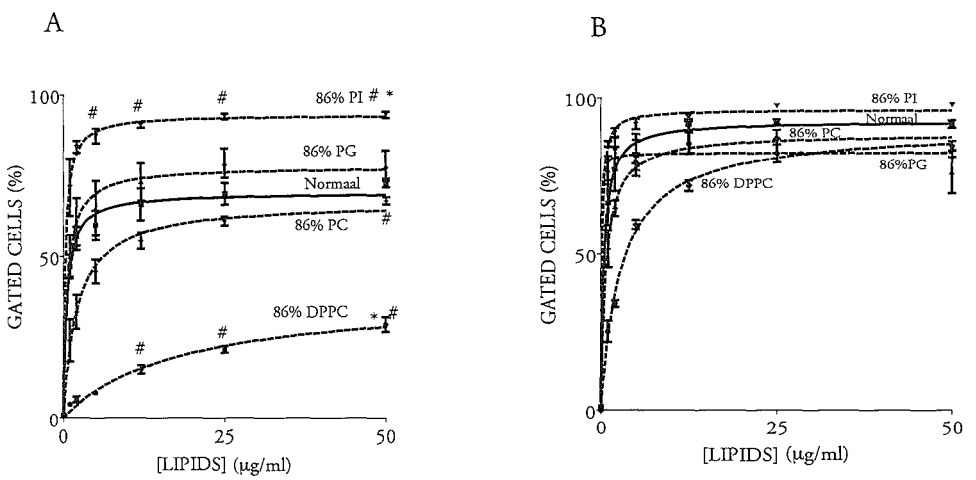

Figure 2. *Concentration-dependent uptake of liposomes*
Alveolar cells type II cells (A) and macrophages (B) were isolated and incubated for 1 hour at 37°C with the same fluorescent liposomes as described in the legend of figure 1. The percentage cells with a cell-associated fluorescence higher than the autofluorescence was determined (= Gated Cells) (n = 4 incubations of $3x10^5$ cells each at every concentration; values expressed as mean ± SEM). # indicates a significant difference compared to the normal liposomes.

Effect of 86% PG Texas Red labeled liposomes on the uptake of 86% PI Bodipy labeled liposomes

The increased uptake of negatively charged PI and PG liposomes combined with an increased percentage alveolar type II cells involved in this uptake, raised the question whether these two types of liposomes are taken up via the same "charge" dependent pathway. Therefore, the effect of 86% PG-Texas Red labeled liposomes on the uptake of 86% PI-Bodipy labeled liposomes by alveolar type II cells and alveolar macrophages was studied by keeping the Bodipy-labeled

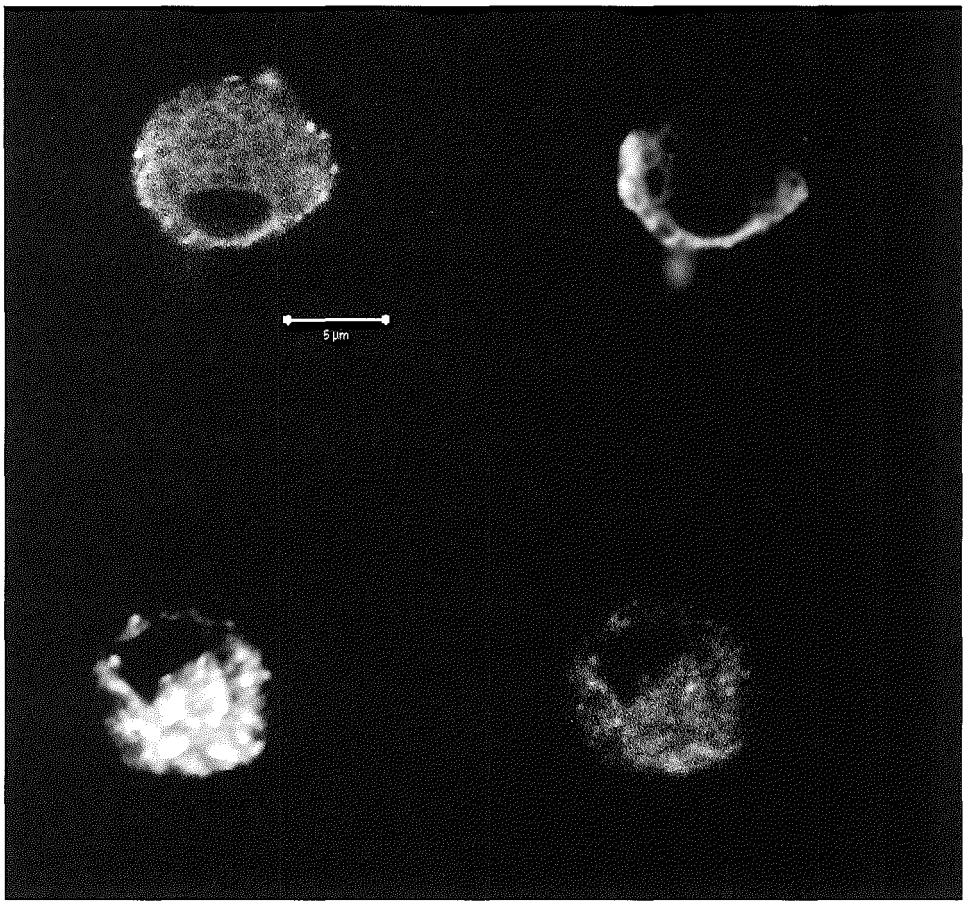

Figure 3. *Localization of cell-associated fluorescence*
Both alveolar type II cells (A, B) and alveolar macrophages (C, D) were isolated and incubated with 86% Bodipy-labeled DPPC liposomes and 86% Texas Red-labeled PG liposomes. One hour after incubation cells were washed 3 times with cold PBS and the cells were mounted on glass coverslips. Confocal laser microscopy demonstrates a punctuate distribution of TexasRed labeled PG liposomes (A, C) and Bodipy labeled DPPC liposomes (B, D) that is not limited to the cell surface, and is not localized in the nucleus of the cell. This distribution of fluorescence throughout the cell indicates internalization of liposomes, strengthened by the fact that untreated cells did not show any fluorescence at the same microscopic settings.

PI liposomes constant and increasing the concentration of added Texas Red labeled PG liposomes. In both cell types, alveolar type II cells and alveolar macrophages, Bodipy-associated mean fluorescence, i.e. uptake of 86% PI liposomes, significantly decreases with an increasing concentration of 86% PG liposomes. On the other hand, the cell-associated Texas Red mean fluorescence, i.e. uptake of 86% PG liposomes, increases significantly with an increasing concentration of 86% PG liposomes (Fig 4 A, B).

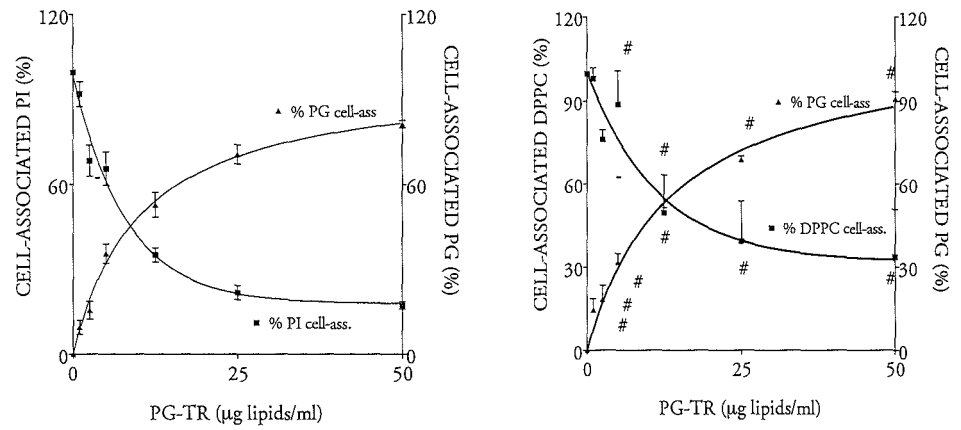

Figure 4. *Competition between PI liposomes and PG liposomes (Mean fluorescence)* Alveolar cells were isolated and incubated for 1 hour at 37°C with 12.5 µg Bodipy labeled PI liposomes (PI:Chol:PE-Bodipy; 86:8:6) and the indicated concentrations of Texas Red labeled PG liposomes (PG:Chol:PE-Texas Red; 86:8:6). The mean cell-associated Bodipy fluorescence (PI; squares) as well as the mean cell-associated Texas Red fluorescence (PG; triangles) was determined in alveolar type II cells (A) and alveolar macrophages (B). Subsequently, the cell-associated Bodipy fluorescence in the presence of PG-Texas Red was expressed as percentage of the cell-associated Bodipy fluorescence of the incubation with 12.5 µg PI-Bodipy alone (Cell-associated PI (%)). The cell-associated Texas Red fluorescence in the presence of PI-Bodipy was expressed as percentage of the cell-associated Texas Red fluorescence of the incubation with 50 µg PG-Texas Red alone (Cell-associated PG (%)). Values are expressed as mean ± SEM (n = 4). # indicates a significant difference compared to 0 µg/ml Texas Red labeled PG liposomes.

In addition, when the concentration of 86% PG liposomes is increased, significantly less cells take up preferentially 86% PI liposomes; there is a significant increase in alveolar cells, either alveolar type II cells or alveolar macrophages, that take up both 86% PI liposomes and 86% PG liposomes (Fig. 5A, B). There are hardly any alveolar cells that internalize only 86% PG liposomes, except at the highest PG concentration, 50 µg/ml, where there is a significant percentage (3%) of the alveolar type II cells that internalize only 86% PG liposomes. The total amount of alveolar cells involved in the uptake does not change significantly (83% for alveolar macrophages and 79% for alveolar type II cells) (Fig 5A, B). These results may indicate that both types of liposomes are taken up via the same pathway. Next, the question rose whether this pathway was the same for DPPC.

Effect of 86% PG Texas Red labeled liposomes on the uptake of 86% DPPC Bodipy labeled liposomes

To determine if 86% DPPC liposomes are taken up via the same pathway as 86% PG, alveolar cells were incubated with 86% DPPC-Bodipy labeled liposomes and increasing concentrations of 86% PG-Texas Red labeled liposomes. Increasing the concentration of 86% PG-Texas Red labeled liposomes results in a significant increase of Texas Red- associated mean fluorescence,

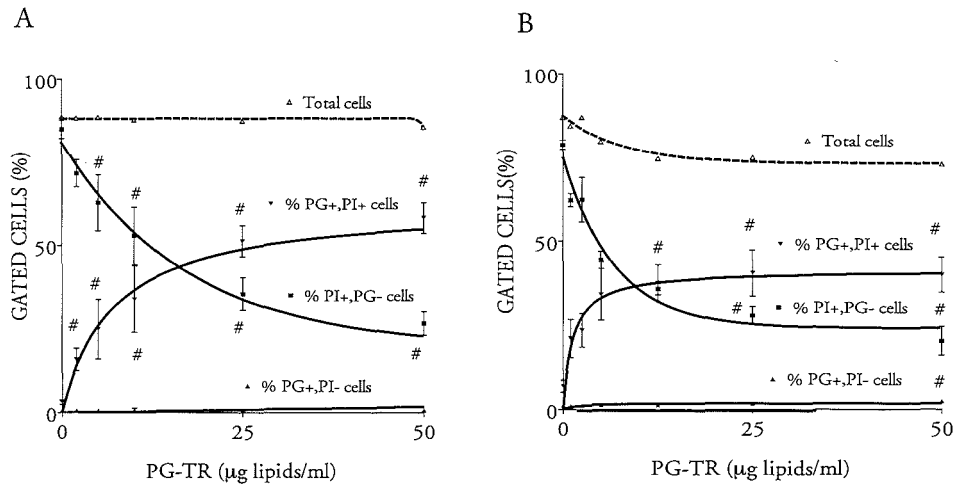

Figure 5. *Competition between PI liposomes and PG liposomes (Gated cells)*
Alveolar cells type II cells (A) and macrophages (B) were isolated and incubated for 1 hour at 37°C with the same fluorescent liposomes as described in the legend of figure 3. The squares (%PI+, PG- cells) are the percentage cells whose Bodipy cell-associated fluorescence is higher than the autofluorescence and whose fluorescence caused by Texas Red is within the range of the autofluorescence. The triangles (%PG+, PI- cells) are the percentage cells that have a Texas Red cell-associated fluorescence higher than the autofluorescence and a Bodipy fluorescence is within the range of the autofluorescence. The circles (%PG+, PI+) are the percentage cells which have a Texas Red cell-associated fluorescence higher than the autofluorescence, as well as a Bodipy cell-associated fluorescence higher than the autofluorescence. The dotted line and asterisks are the total percentage of cells with a fluorescence higher than the autofluorescence, either by Bodipy or Texas Red. Values are expressed as mean ± SEM (n = 4). # indicates a significant difference compared to 0 µg/ml Texas-Red labeled PG liposomes.

together with a significant decrease in Bodipy-associated mean fluorescence (Fig. 6A, B). Raising the concentration of 86% PG liposomes results in a significant increase in double labeled alveolar macrophages, together with a significant decrease in Bodipy labeled alveolar macrophages.
At a concentration of 12.5 µg/ml 86% PG liposomes, the amount of double labeled alveolar macrophages decreases again and the amount of alveolar macrophages that take up only 86% PG liposomes increases (Fig. 7B). The alveolar type II cells demonstrate a similar change in cells involved in the uptake, first the amount of double labeled cells increase, and at a concentration of 5 µg/ml, which is lower than for alveolar macrophages, the amount of double alveolar type II cells decreases, together with an increase in alveolar type II cells that take up only 86% PG liposomes (Fig 7A).

Effect of 86% DPPC Texas Red labeled liposomes on the uptake of 86% PG Bodipy labeled liposomes
To determine whether 86% DPPC liposomes can inhibit the uptake of 86% PG liposomes, suggesting a similar pathway for uptake of liposomes, alveolar cells were incubated with 86%

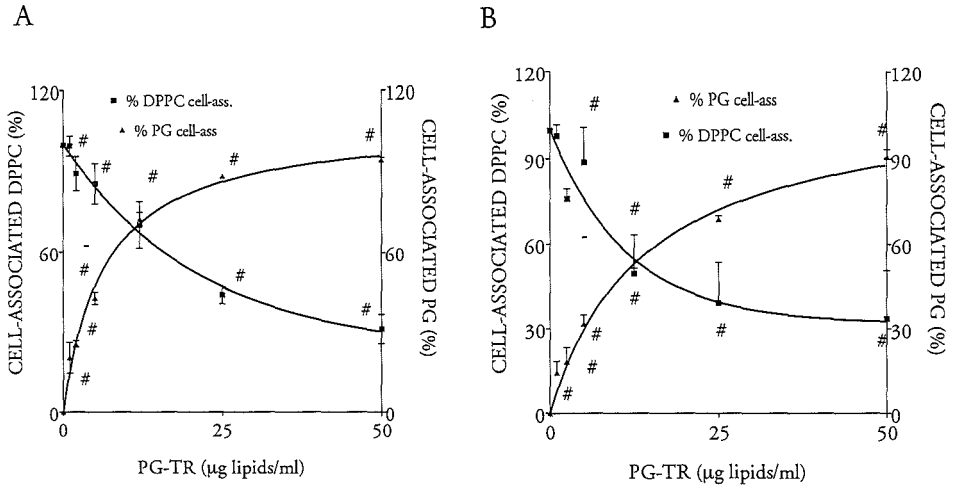

Figure 6. *Uptake of DPPC liposomes in presence of Texas-Red labeled PG liposomes (Mean fluorescence)*
Alveolar cells were isolated and incubated for 1 hour at 37°C with 12.5 µg Bodipy labeled DPPC liposomes (DPPC:Chol:PE-Bodipy; 86:8:6) and the indicated concentrations of Texas Red labeled PG liposomes (PG:Chol:PE-Texas Red; 86:8:6). The mean cell-associated Bodipy fluorescence (DPPC; squares) as well as the mean cell-associated Texas Red fluorescence (PG; triangles) was determined in alveolar type II cells (A) and alveolar macrophages (B). Subsequently, the cell-associated Bodipy fluorescence in the presence of PG-Texas Red was expressed as percentage of the cell-associated Bodipy fluorescence of the incubation with 12.5 µg DPPC-Bodipy alone (%Cell-associated DPPC). The cell-associated Texas Red fluorescence in the presence of DPPC-Bodipy was expressed as percentage of the cell-associated Texas Red fluorescence of the incubation with 50 µg PG-Texas Red alone (% Cell-associated PG). Values are expressed as mean ± SEM (n = 4). # indicates a significant difference compared to 0 µg/ml Texas-Red labeled PG liposomes.

PG-Bodipy labeled liposomes and increasing concentrations of 86% DPPC-Texas Red labeled liposomes. Increasing the concentration of 86% DPPC-Texas Red labeled liposomes results in a significant decrease of Bodipy-associated mean fluorescence per alveolar type II cell, but no changes were seen in cell-associated fluorescence per alveolar macrophages (Table 1).
Increasing the concentration of 86% DPPC liposomes results in a significant decrease in single-labeled, Bodipy associated, alveolar cells, and a concomitant increase in double-labeled, Bodipy and Texas-Red associated, alveolar cells. There is a slight, non-significant, increase in alveolar cells that are only Texas-Red labeled and thus took up only 86% DPPC liposomes.

Discussion

In the current study we investigated the uptake of the main phospholipid components of surfactant in vitro by alveolar type II cells and alveolar macrophages. We used fluorescent labeled liposomes containing 86% of the studied component, 8% cholesterol and 6% labeled

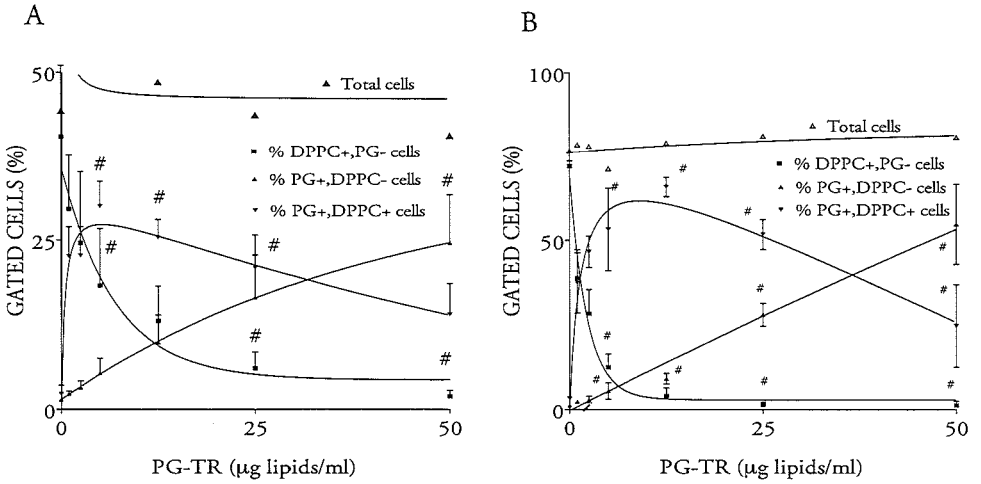

Figure 7. *Uptake of DPPC liposomes in presence of Texas-Red labeled PG liposomes (Gated cells)*
Alveolar cells type II cells (A) and macrophages (B) were isolated and incubated for 1 hour at 37°C with the same fluorescent liposomes as described in the legend of figure 5. The squares (%DPPC+, PG- cells) are the percentage cells which have a Bodipy cell-associated fluorescence higher than the autofluorescence and a Texas Red fluorescence not higher than the autofluorescence. The triangles (%PG+, DPPC- cells) are the percentage cells which have a Texas Red cell-associated fluorescence higher than the autofluorescence and a Bodipy fluorescence not higher than the autofluorescence. The circles (%PG+, DPPC+) are the percentage cells which have a Texas Red cell-associated fluorescence higher than the autofluorescence as well as Bodipy cell-associated fluorescence higher than the autofluorescence. The dotted line and asterisks are the total percentage of cells with a fluorescence higher than the autofluorescence, either by Bodipy or Texas Red. Values are expressed as mean ± SEM (n = 4). # indicates a significant difference compared to 0 µg/ml Texas-Red labeled PG liposomes.

phosphatidylethanolamine. Confocal laser microscopy demonstrated the intracellular presence of cell-associated fluorescence, indicating that the fluorescent labeled liposomes are indeed taken up by the alveolar cells (Fig. 3). The uptake of the liposomes shows a concentration-dependent increase in cell-associated fluorescence (Fig. 1), reaching a plateau at 25 µg/ml, similar to the results of our previous study[11]. The uptake of the more negatively charged liposomes, i.e. 86% PG and 86% PI liposomes, by alveolar type II cells and alveolar macrophages is significantly higher than that of the more neutrally charged liposomes, i.e. 86% PC, 86% DPPC and normal liposomes. These results indicate that the alveolar cells have a higher affinity for negatively charged liposomes.
The mechanism of internalization of liposomes by the alveolar cells is not clear at the moment. For macrophages as well as alveolar type II cells at least part of the uptake of liposomes seems to be mediated by the coated-pit pathway[14-16], suggesting a receptor-mediated process, but for which the receptor and the specificity of the receptor are unknown. For arguments sake we assume that the uptake of liposomes by alveolar cells is receptor mediated. A receptor with a high affinity for negatively charged liposomes per se and which does not discriminate between

Table 1. *Uptake of PG liposomes in the presence of increasing concentrations DPPC liposomes*

110 µg/ml PG	Fluorescence PG (%)		PG+ cells (%)		DPPC+ cells (%)		PG+ DPPC+ cells (%)	
DPPC (µg/ml)	ATII	AM	ATII	AM	ATII	AM	ATII	AM
0	100	100	83	81	0	0	5	4
10	86	90	63*	47*	0	1	14	37*
25	59	84	52*	25*	1	1	20	53*
50	63	81	43*	15*	1	2	27	62*
100	56*	78	36*	13*	5	5	26	59*

Isolated alveolar cells were incubated with 10 µg/ml Bodipy labeled PG and increasing concentration TexasRed labeled DPPC. Mean cell-associated fluorescence per cell was determined and expressed as percentage of mean fluorescence in absence of DPPC (Fluorescence PG (%)).
Definition of abbreviation: ATII, alveolar type II cells; AM, Alveolar macrophages; PG+ cells, Bodipy fluorescence associated positive cells; DPPC+cells, TexasRed fluorescence associated positive cells; PG+DPPC+ cells, both Bodipy and TexasRed fluorescence associated positive cells. (* $p<0.05$).

PG and PI liposomes, as was shown by competition experiments with 86% PI and 86% PG containing liposomes.
The receptor is not specific for negatively charged liposomes. The competition experiments of PG and DPPC liposomes demonstrate that the uptake of DPPC by the alveolar cells can be fully blocked by PG leading to uptake of PG instead, whereas the other way around, competing PG with DPPC, leads to only a partial reduction in the PG uptake by alveolar cells (Table 1). Therefore the receptor affinities for DPPC and PG and/or PI liposomes seem to differ; the highest affinity being for PG followed by PI and DPPC. There seems to be very few differences in the uptake of phospholipids by alveolar macrophages and alveolar type II cells, but from our experiments it can not be concluded that the "phospholipid receptor" of alveolar macrophages and alveolar type II cells is the same. The only striking difference is the number of cells involved in the uptake of DPPC liposomes. The number of alveolar type II cells that internalize DPPC liposomes is significantly lower than the number of alveolar macrophages (29% versus 72%), whereas the number of cells involved in the uptake of PG liposomes is approximately the same for alveolar macrophages and type II cells (75-80%). A possible explanation can be that the distribution of receptors on alveolar macrophages and type II cells differs. Most of the alveolar macrophages may have a high density of receptors, whereas the alveolar type II cell population consists of several subpopulations of cells with different receptor densities. Hence, adding DPPC liposomes to alveolar cells will lead to association of DPPC with practically all macrophages and, in the case of type II cells, association will occur only with the subpopulation of type II cells with the highest receptor density. PG or PI liposomes will also associate with the subpopulations of alveolar type II cells with lower receptor densities due to the high affinity of the receptor for these two phospholipids. This hypothesis may explain the competition experiments of DPPC with PG and vice versa using alveolar type II cells (Figs. 6, 7 and Table 1). By competing DPPC with PG a rapid drop in the cell-associated DPPC is observed, whereas competing PG with DPPC only a slight reduction in

the cell-associated PG is observed which is indicative for a higher affinity of the alveolar cells for PG than for DPPC. The maximal number of alveolar type II cells that can internalise DPPC is approximately 30%, adding PG to the incubation results in a rapid decline in the number of cells that contain only DPPC with a concomitant increase in PG+ cells. The double positive cells increase initially, but at higher PG concentrations the number decreases. Apparently, at relatively low PG concentrations, PG competes with DPPC for the cells with the highest receptor density leading to cells which internalise both lipids as well as small numbers of PG+ and DPPC+ cells. At higher PG concentrations, the PG uptake by the cells with the high receptor density is saturated, leading to the uptake of PG by the subpopulation of cells with the low receptor density, the cells which cannot internalise DPPC. Therefore, there are more alveolar type II cells that can take up PG, which consist of the subpopulations of cells with the high as well as with the low receptor densities, and less cells which can internalise DPPC, namely the subpopulation with the high receptor density. The results of the competition between PG and DPPC can be explained in a similar way. In the absence of DPPC the number of PG+ cells is approximately 80%, which consist of the subpopulations with high as well as low receptor densities. Including DPPC in the incubations leads to a reduction in the number of the PG+ cells that stabilizes at 30-40% PG+ cells at a 10-fold higher concentration of DPPC over PG. This cell population reflects the low receptor density cells. Hence, about 40-50% of the alveolar type II cells have a low receptor density and cannot internalise DPPC, and about 30-40% of the alveolar type II cells have a high receptor density and are able to internalise both DPPC and PG.

Up to now, the focus has been on the role of the surfactant proteins in the uptake of DPPC by alveolar cells in vitro. At least three of the four surfactant proteins, SP-A, SP-B and SP-C, were implicated in the internalization of surfactant lipids by alveolar cells. SP-A and SP-C were shown to stimulate the uptake of liposomes by alveolar cells[6-9, 16, 17], whereas the influence of SP-B on the uptake of liposomes is more complex. SP-B was reported to stimulate the uptake by alveolar cells[18-20], whereas others have shown that SP-B can inhibit the SP-C mediated uptake by alveolar type II cells[7, 8]. *In vivo* the role of the surfactant proteins becomes more complex and unclear. In SP-A knock out mice the uptake of surfactant by alveolar cells appears to be normal[21, 22], whereas one would expect that based on the vitro results the uptake would be disturbed in these animals, only under inflammatory conditions are differences in lipid uptake between SP-A (+/+) and SP-A (-/-) mice observed[23]. In contrast, for SP-D no influence on lipid uptake could be demonstrated in vitro[17], while the surfactant lipid metabolism in SP-D (-/-) mice was disturbed[24, 25]. Therefore, in spite of numerous studies, the role of the surfactant proteins in the surfactant lipid metabolism in vivo still needs to be further clarified. The influence of the surfactant phospholipid composition, in particular the charge of the phospholipids, on the uptake of surfactant liposomes by alveolar cells is also not clear. The first data are derived from in vivo experiments by Jacobs et al.[26, 27], demonstrating the disappearance of radiolabeled PG in the lungs of neonatal and adult rabbits after intratracheal injection. Bates and Chandler have shown that radiolabeled PG containing vesicles are internalized more rapidly by alveolar type II cells in vitro[28, 29]. Recently, a study by Quintero et al. indicated that alveolar macrophages internalize PG more readily than DPPC in vitro[10]. The present study indicates that, besides alveolar macrophages, also alveolar type II cells have a higher affinity for PG or PI containing liposomes than for the more neutrally charged liposomes. In addition, the pathway of uptake of PG, PI, and DPPC by alveolar cells appears to be the same. Uptake of surfactant lipids by alveolar cells is an important step in the recycling of surfactant. Surfactant recycling itself is crucial to maintain normal lung functions. Therefore, changes in the surfactant lipid composition, for instance, more or less PG or other negatively charged lipids, may not only alter the uptake of these lipids but also of DPPC, the most important surface tension lowering component of surfactant. In various diseases, such as respiratory distress

syndrome (RDS), extrinsic allergic alveolites and respiratory infections changes in the surfactant lipid composition were observed[30, 31]. Whether an altered uptake of surfactant lipids by alveolar cells plays a role in the pathogenesis of respiratory diseases is not yet known. Further studies on the role of the surfactant lipid composition on the uptake of surfactant by alveolar cells are therefore warranted. In particular, the influence of the lipid composition on the in vivo uptake of surfactant and the effects on lung mechanics. Not only will this information expand our knowledge on the surfactant metabolism but it might also provide new tools for therapeutic interventions for various respiratory diseases.

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CHAPTER 4

Influence of phosphatidylglycerol on the uptake of liposomes by alveolar cells and or lung function

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Submitted

Abstract

The effect of phosphatidylglycerol on the uptake by alveolar type II cells and alveolar macrophages as well as the effect on endogenous surfactant function was studied *in vivo*. Healthy ventilated rats were intratracheally instilled with fluorescent labeled liposomes with different concentrations phosphatidylglycerol. Lung function was determined by monitoring arterial oxygenation and, at the end of the experiment, by static pressure-volume curves. In addition, alveolar cells were isolated and cell-associated fluorescence was determined using flow cytometry. The results show that in the presence of cofactors (Ca^{2+}, Mg^{2+}) phosphatidylglycerol stimulates the uptake by alveolar macrophages, but hardly affects the uptake by alveolar type II cells. High concentrations phosphatidylglycerol reduce the amount of alveolar macrophages in the alveolar space and induce a deterioration of lung function. On the other hand, the presence of cofactors protects the lung against the negative effects of phosphatidylglycerol on endogenous surfactant and alveolar macrophages.
This study indicates that the phosphatidylglycerol concentration may play a fundamental role in the surfactant function and metabolism; though further investigations are needed to clarify the mechanism.

Introduction

The alveolus is lined with a thin layer of lipids and proteins, called surfactant, and this surface active agent has an essential role in maintaining normal lung function. Pulmonary surfactant is produced by alveolar type II cells. By weight, approximately 90% of surfactant consists of lipids. Although, the lipid composition varies in different species, its major component is phosphatidylcholine (PC; 70-80%) of which nearly 50% is saturated dipalmitoylphosphatidylcholine (DPPC). DPPC is the major surface tension-reducing component of surfactant. In addition, surfactant contains variable amounts of phosphatidylglycerol (PG; 7-18%), phosphatitylinositol (PI; 2-4%) and phosphatidylethanolamine (PE; 2-3%)[1].
The remaining percentage of lipids consists of other phospholipids and cholesterol[1]. The additional 10% consists of surfactant specific proteins (SP), SP-A, SP-B, SP-C and SP-D.
It has been demonstrated that these surfactant proteins, although only a small part of surfactant, play an essential role in the homeostasis of surfactant. This homeostasis consists mainly of production, secretion, formation of lipid monolayer, turnover, re-uptake and recycling. Especially SP-A has been shown to affect the uptake of surfactant by alveolar cells[2-5]. However, recent *in vitro* studies have demonstrated that, aside from these surfactant proteins, the different lipid components also affect the uptake[6, 7]. In the past significant differences in lipid composition between adults and neonates have been described[8-11]. In addition, the use of exogenous surfactant is now widely accepted as a treatment of surfactant dysfunction in neonates and is even under consideration as a therapy in adults[12]. Considering the natural differences in surfactant lipid composition between adults and neonates, as well as the fact that neonates rely more on recycling of surfactant than adults[13, 14], the role of the single lipid components on the uptake of surfactant by alveolar cells has to be elucidated.
Phosphatidylglycerol is the second major component after PC, and *in vitro* studies have demonstrated a significant effect of phosphatidylglycerol on the uptake[6, 7]. However, extrapolation of the results of *in vitro* experiments regarding the uptake of liposomes to the *in vivo* situation is not always valid, since we were able to demonstrate significant differences in uptake of surfactant-like liposomes *in vitro* in comparison to *in vivo*[15]. Therefore, in the current study the effect of different concentrations phosphatidylglycerol incorporated in the liposome on the uptake was determined in mechanically ventilated rats using flow cytometry. In addition, we also investigated possible effects of these different concentrations on lung mechanics.

Materials and methods

Ethical Guidelines
This study was approved by the Institutional Animal Committee at the Erasmus MC Rotterdam. The studies were performed in 72 male Sprague-Dawley rats (IFFA Credo, The Netherlands) with a bodyweight of 310 ± 10 g.

Materials
Dipalmitoylphosphatidylcholine (DPPC), egg phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and cholesterol were purchased from Sigma (Zwijndrecht, The Netherlands). Head-group rhodamine labeled phosphatidylethanolamine (PE) (Rhodamine DHPE) was obtained from Molecular Probes (Leiden, The Netherlands).

Liposome preparation
To prepare liposomes, the different lipids (Sigma, Zwijndrecht, The Netherlands) were mixed at the indicated weight ratio (Table 1). The mixture of lipids was dried under a stream of nitrogen gas. The liposomes were either suspended in PBS or solution 2 (140 mM NaCl, 5 mM KCl, 2.5 mM phosphate buffer, 10 mM HEPES, 6 mM glucose, 2.0 mM $CaCl_2$ and 1.3 mM $MgSO_4$), to a concentration of 1.0 mg lipids/ml using glass pearls and vortexing.

Table 1. *Composition of the fluorescent labeled liposomes*

	0% PG	Normal (8% PG)	20% PG	40% PG	60% PG	86% PG
DPPC	61	55	46	32	17.4	0
PC	23	21	18	12	6.6	0
PG	0	8	20	40	60	86
PI	2	2	2	2	2	0
PE (labeled)	6	6	6	6	6	6
Cholesterol	8	8	8	8	8	8

Weight ratio of the individual lipids used for the composition of the different liposomes; DPPC: dipalmitoylphosphatidylcholine; PC: phosphatidylcholine; PG: phosphatidylglycerol; PI phosphatidylinositol; PE: phosphatidylethanolamine.

Immediately prior to use, the liposome suspension was sonicated for 2 min on ice using an ultrasonic disintegrator (Branson Sonifier 250, Danbury, USA) to prepare small unilamellar liposomes[15]. The size of the liposomes was determined by dynamic light scattering at 25°C with a Malvern 4700 system using a 25 mW He-Ne laser (NEC, Tokyo, Japan) and the auto measure version 3.2 software (Malvern Ltd, Malvern, UK). As a measure of particle size distribution of the dispersion, the system reports a polydispersity index (p.d.). This index ranges from 0.0 for a monodisperse and up to 1.0 for an entirely polydisperse dispersion. After ultrasonification the liposomes size ranged from 140 to 165 nm and the polydispersity index ranged from 0.2 to 0.35.

Intratracheal instillation of fluorescent liposomes
After induction of anesthesia with a mixture of nitrous oxide (66%), oxygen (33%) and isoflurane (1-2%) a sterile polyethylene catheter (0.8 mm o.d.) was inserted into one of the carotid arteries. The animals were then tracheotomized and a sterile metal cannula was inserted into the trachea.
After these surgical procedures, gaseous anesthesia was ended and replaced with an intraperitoneal injection of pentobarbital sodium (60 mg/ml, Nembutal®, Algin BV, Maassluis, The Netherlands) at a dose of 30 mg/kg bodyweight every hour.
Muscle relaxation was induced and maintained by an hourly intramuscular injection of pancuronium bromide (2 mg/kg, Pavulon; Organon Technika, Boxtel, The Netherlands). The animals were then mechanically ventilated with a Servo® ventilator 900 (Siemens-Elema, Solna, Sweden) set to pressure control mode using a frequency of 30/min, an inspiratory/expiratory ratio of 1:2, a positive end-expiratory pressure (PEEP) of 2 cm H_2O, a peak inspiratory pressure (PIP) of 12 cm H_2O and FiO_2 was set to 1.
Before instillation of the labeled liposomes, PEEP was increased to 6 cm H_2O and PIP was increased to 26 cm H_2O. After disconnection from the ventilator, the liposomes were administered intratracheally as a bolus of 3 ml/kg followed by a bolus of air (12 ml/kg), directly into the endothracheal tube via a syringe, and the animals were immediately reconnected to the ventilator. Thirty min after instillation of the liposomes PEEP was reduced to 2 cm H_2O and PIP to 12 cm H_2O.
Arterial blood gases were measured with conventional methods (ABL 555, Radiometer, Copenhagen, Denmark) at start of ventilation, immediately after instillation of the liposomes and every 30 minutes thereafter. One hour after ventilation the animals were sacrificed by exsanguination via the abdominal aorta and the alveolar cells were isolated to determine the cell-associated fluorescence. Control animals were sacrificed immediately after anesthesia and their isolated alveolar type II cells and alveolar macrophages were used to correct for auto fluorescence.

Isolation of alveolar type II cells and alveolar macrophages
Prior to isolation of the cells, the thorax was opened and the blood cells were removed from the lungs by perfusing the pulmonary artery with saline (37°C) supplemented with 20 IE heparin per ml (Leo Pharma, Weesp, The Netherlands). The lungs were removed from the thoracic cavity en bloc and lavaged with 10 ml of solution 1 [140 mM NaCl, 5 mM KCl, 2.5 mM $Na_2HPO_4.2H_2O$, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethaesulfonic acid), 6 mM glucose, 0.2 mM EGTA (ethylene glycol-bis- (β-amino ethyl ether) N,N'-tetra-acetic acid), pH 7.40] at 22°C. This procedure was repeated four times. The lung lavages were pooled per animal and centrifuged (100x *g*; 10 min; 4°C). The cellular pellet, i.e. alveolar macrophages, were suspended in solution 2 to a concentration of $2x10^6$ cells/ml and stored on ice until further use. The alveolar type II cells were isolated according to Dobbs et al.[16]. Alveolar type II cells were suspended in solution 2 at a concentration of $2x10^6$ cells/ml and stored on ice until further use. Alveolar macrophages were identified using monoclonal antibodies specific for rat macrophages (ED9) and alveolar type II cells were identified using an alkaline phosphatase assay as described by Edelson et al.[17]. The average yield of alveolar type II cells was $16x10^6$ a purity of 81 ± 4% and $5x10^6$ alveolar macrophages with a purity of 90 ± 6% per rat.

Flow cytometry
Cell-associated fluorescence of the alveolar type II cells and alveolar macrophages as a measure for internalized liposomes was determined using flow cytometry (FACSCalibur; Becton Dickinson; Mountain View, USA). The cell-associated fluorescence of 15,000 cells was deter-

mined. Alveolar macrophages and alveolar type II cells derived from control animals were used in each experiment to determine the auto fluorescence of the cells. Subsequently, the mean cell-associated fluorescence was determined only for those cells that had a higher fluorescence than that caused by auto-fluorescence (gated cells).

Determination of lung mechanics

Due to severe deterioration in blood gas values in the animals receiving 86% PG liposomes, additional experiments were performed to study the effect of these liposomes on lung mechanics. A total of 36 animals were subjected to procedures identical to those described above (ventilation and instillation of the liposomes) and after 1 hour the animals were sacrificed by an overdose of pentobarbital (60 mg/kg). Next, thorax and diaphragm were opened (to eliminate the influence of chest wall compliance and intra-abdominal pressure) and a static pressure-volume (P/V) curve from the lungs was recorded using conventional techniques[18]. Maximal compliance (C_{max}) was defined as the steepest part of the P/V deflation curve, and was determined for each animal. The Gruenwald index, which characterizes the surfactant system *in situ*[19], was calculated from the P/V curve, defined as $(2V_5+V_{10})/2V_{max}$, where V_5, V_{10} and V_{max} are the lung volumes at transpulmonary pressures of respectively 5, 10 and 35 cm H_2O from the deflation limb. Thereafter, broncho-alveolar lavage (BAL) was performed with saline (30 ml/kg heated to 37°C) five times and its percentage recovery was calculated. Cell debris was removed from the BAL by centrifugation at 400 x *g* for 10 min. The protein concentration of the BAL fluid was determined using the Bradford method (Bio-Rad protein assay, Munich, Germany) with bovine serum albumin (Sigma, St Louis, MO, USA) as a standard[20].

Histological sampling

To study the presence of histological changes in lung morphology, one hour after instillation of the liposomes, blood was removed from the lungs by perfusion of the pulmonary arteries with warmed saline (37°C) supplemented with 20 IE heparin per ml (Leo Pharma, Weesp, The Netherlands). Next, the lungs were fixated as previously described (17). Briefly, after perfusion the lungs were fixated with a fixation solution, consisting of 3.6% formaldehyde and 0.25% glutaraldehyde. Prior to fixation, the airway pressure was momentarily increased to 10 cmH_2O and thereafter maintained at 10 cmH_2O. Blocks of tissue were taken from the center of the upper and middle lobe and from the ventral and dorsal part of the lower lobe. The specimens were embedded in paraffin, sectioned and stained with hematoxylin and eosin and lung morphology was analyzed using light microscopy.

Statistical analysis

Differences in blood gas values over time and differences between the groups that received liposomes with a different composition were analyzed using a repeated measurement ANOVA followed by a Bonferroni post-hoc test. Differences in mean fluorescence per cell and percentage of cells involved in the uptake (gated cells) were analyzed using ANOVA followed by a Bonferroni post-hoc test, as were differences in maximal compliance, Gruenwald index, maximal volume at 35 cm H_2O, protein concentration in the BAL and number of macrophages retrieved by BAL. Differences were considered statistically significant at a $p < 0.05$. Values are expressed as mean ± SD.

Results

Influence of the phosphatidylglycerol content of fluorescent labeled liposomes on the uptake
As described previously, incorporation of a higher concentration of phosphatidylglycerol in the labeled liposomes increases the uptake of these liposomes by alveolar macrophages and alveolar type II cells *in vitro*[7]. To determine if these effects of phosphatidylglycerol on the uptake are also present *in vivo*, ventilated rats were intratracheally instilled with fluorescent labeled liposomes that contained various concentrations phosphatidylglycerol. One hour after instillation of these liposomes, the animals were sacrificed and alveolar cells were isolated. The uptake of liposomes by alveolar type II cells decreased significantly when the amount of PG was increased to 20% in comparison to the uptake of normal liposomes, containing only 8% PG. However, further increment of the amount of PG did not have a significant effect on the uptake of these liposomes by alveolar type II cells in comparison to the 'normal' liposomes;

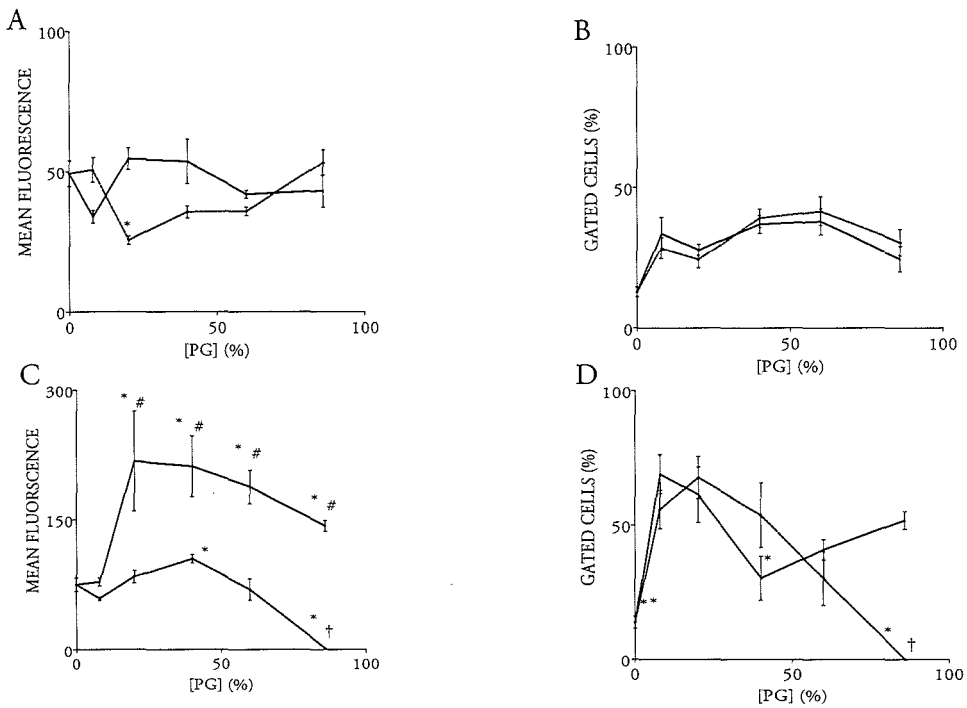

Figure 1. *Effect of different concentrations PG on the uptake*
Fluorescent labeled liposomes with different concentrations of PG, either suspended in PBS (dotted line) or solution 2 (solid line), were intratracheally instilled in mechanically ventilated rats (3 mg/kg). One hour after instillation alveolar type II cells were isolated and the mean cell-associated fluorescence of gated alveolar type II cells (A) and alveolar macrophages (C) was determined. In addition, the percentage gated alveolar type II cells (B) and alveolar macrophages (D) was determined (n = 4 rats at each concentration; values are mean ± SEM). * indicates a significant difference ($p < 0.05$) in comparison to the normal (8% PG) liposomes. # indicates a significant difference ($p < 0.05$) in comparison to the same liposomes resuspended in PBS. †, at a concentration of 86% PG liposomes no macrophages were detected in the bronchoalveolar lavage.

however, the uptake tends to increase compared to the uptake of 20% PG liposomes (Fig. 1A). When the liposomes are resuspended in solution 2, containing cofactors known to effect the uptake (e.g. calcium and magnesium) no significant differences are observed in comparison to the same liposomes resuspended in PBS. The number of alveolar type II cells involved in the uptake is unaffected by the amount of PG within the liposome in comparison to the normal liposomes (Fig 1B).
As surfactant-like liposomes are taken up by both alveolar type II cells and alveolar macrophages, the effects of the concentration PG on the uptake was also studied in alveolar macrophages. An increase in the amount of PG within the liposomes results in an increased uptake, with an optimum at 40% PG, where the increase is significantly higher in comparison to normal liposomes containing 8% PG. When the PG concentration is raised to 60% no significant differences with normal liposomes are observed; however, further increment of the amount PG within the liposomes (86%) results in an absence of alveolar macrophages in the BAL and thus no cell-associated fluorescence can be determined with the use of 86% PG liposomes (Fig. 1C). Increasing the amount of PG tends to increase the amount of macrophages taking part in the uptake with an apparent optimum at 20%, although this increase is not significant. Higher PG concentrations result in a decline of the number of cells involved in the uptake accumulating at a PG concentration of 86% in the total absence of alveolar macrophages in the lung lavage.
However, suspending the liposomes in solution 2 instead of PBS, as was done in the previous experiment, an increase in the amount of PG within the liposomes results in an enhanced uptake for all concentrations with a possible optimum at 20% PG in comparison to normal liposomes (8% PG); though this increased uptake is not significant. Also, the number of alveolar macrophages involved in the uptake depends on the PG concentration. An apparent optimum is observed at 20%, increasing the concentration PG results in a gradual decrease in the number of cells which internalize liposomes (Fig 1D).

Alveolar macrophages and protein concentration in BAL
As mentioned previously, the presence of high amounts of PG, i.e. 86%, in the liposomes leads to an absence of alveolar macrophages in the BAL. Therefore, we were interested at which concentration of PG this effect occurs. However, when the amount of PG is increased to a concentration as high as 20%, the number of alveolar macrophages already decreases significantly. This decrease is also observed for the liposomes with 40 and 60% PG incorporated.
However, with the exception of liposomes containing 60% PG, when the liposomes are resuspended in solution 2, normal amounts of alveolar macrophages are found in BAL of animals instilled with the different liposomes (Fig. 2A). The BAL which were performed at the end of the experiment to collect alveolar macrophages were also used to determine protein-concentration in the BAL. The influx of protein in the lungs is increased when the PG concentration in the liposomes is raised for liposomes suspended in PBS, whereas no effect on the protein content of the BAL is observed when liposomes are suspended in solution 2 (Fig. 2B).

Histological changes with 86% phosphatidylglycerol liposomes
As mentioned above, no alveolar macrophages could be retrieved by alveolar lavage when the liposomes were suspended in PBS. To study whether alveolar macrophages were indeed absent in the alveolar space when the lungs were instilled with 86% PG liposomes, histological samples of the lungs of these animals were obtained and analyzed using light microscopy. The samples were acquired from animals instilled intratracheally with normal (8% PG) liposomes and animals that had received 86% PG liposomes. The histological samples from the latter animals showed patchy interstitial infiltrate causing severe wall thickening indicating early

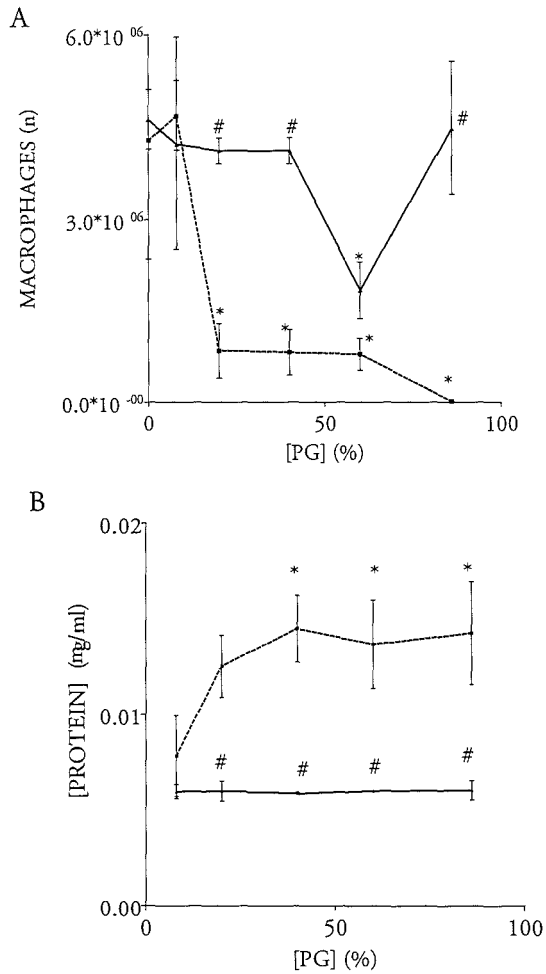

Figure 2. *Effect of different concentrations PG on lavage parameters*
One hour after instillation of the fluorescent labeled liposomes, either suspended in PBS (dotted line) or solution 2 (solid line), the rats were sacrificed and bronchoalveolar lavages were obtained. After centrifuging the lavages at 100xg the number of alveolar macrophages in the pellet (A) and the amount of proteins in the supernatant was determined (B) (n = 4 rats at each concentration; values are mean ± SEM). * indicates a significant difference ($p < 0.05$) in comparison to the normal (8% PG) liposomes. # indicates a significant difference ($p < 0.05$) in comparison to same liposome resuspended in PBS.

diffuse alveolar damage. In addition no alveolar macrophages could be detected within the alveolar space (Fig. 3).

Lung mechanics after instillation of fluorescent labeled liposomes with a different concentration phosphatidylglycerol

During the experiment, blood gas values were determined to study the effects of the liposomes on the arterial oxygenation. Instillation of 86% PG liposomes results in a significant decrease over time and, after 1 hour, the arterial blood gas values meet international criteria for acute respiratory failure[21].

This dramatic effect of PG liposomes is concentration-dependent, because increasing the amount of PG within the liposomes leads to a concomitant decrease in arterial oxygenation (Fig. 4). However, when the liposomes are resuspended in solution 2 no significant deterioration in alveolar oxygenation is observed.

To determine if this dramatic decrease in gas-exchange is caused by a diminished surfactant function, static P/V curves were performed at the end of the experiment. The Gruenwald

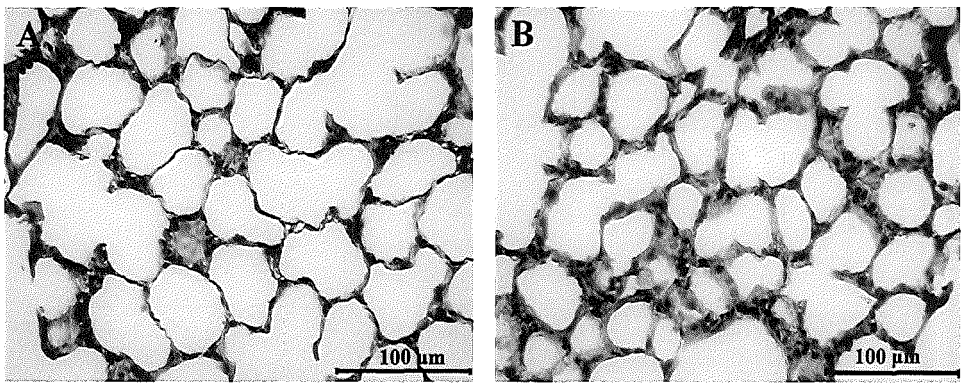

Figure 3. *Slices of the left lung lobe after instillation of liposomes*
Representative coupes of the left lobe of an animal instilled with normal liposomes (A) and of an animal instilled with 86% PG liposomes (B). Lung slices were H stained and are shown at 400x magnification.

index, as a measure of lung stability[19], demonstrates significantly lower values for all liposomes with a PG content higher than 8% suspended in PBS (Fig. 5A). When the liposomes are resuspended in solution 2 no significant decrease is present, except for the liposomes containing 60% PG (Fig 5A).
These results are corroborated by the changes in maximal compliance; instillation of liposomes with a PG concentration higher than the physiological concentration of 8%, and suspended in PBS results in a significant decrease of the maximal compliance (Fig. 5B). However, when the liposomes are resuspended in solution 2, only for 60% and 86 % PG liposomes are significant change observed. The maximal lung volume at 35 cm H_2O is not affected by the amount of PG within the liposomes nor by the solution the liposomes are resuspended in (Fig. 5C).

Discussion

Pulmonary surfactant is essential for normal physiological lung function. To maintain a low variable surface tension, surfactant forms a lipid monolayer, lining the alveolo-capillary membrane. As a result of normal breathing, the radius of the alveolus increases and decreases, causing lipids to be squeezed out of the lipid monolayer during expiration. In addition, the surfactant in the alveolus is turned over from active into inactive surfactant. In other words, the amount of surface-active surfactant within the alveolus would decrease if the alveolar type II would not compensate the loss, by secreting pulmonary surfactant. As *de novo* synthesis of surfactant has been suggested to be insufficient to compensate for the constant "loss" of active surfactant, alveolar type II cells rely on recycling inactivated surfactant[13, 14]. To ensure recycling, the inactivated surfactant is cleared from the alveolar space by alveolar type II cells and in addition inactive surfactant is also cleared by alveolar macrophages[13].
This process of uptake or re-uptake of surfactant lipids is thought to be essential in the surfactant metabolism. Both alveolar type II cells as well as alveolar macrophages have been demonstrated to take up surfactant, though their relative contribution to the clearance remains under discussion as results are somewhat contradictory[22-24]. However, most of the studies have focused on the uptake of surfactant's main component DPPC, or on the effects of the

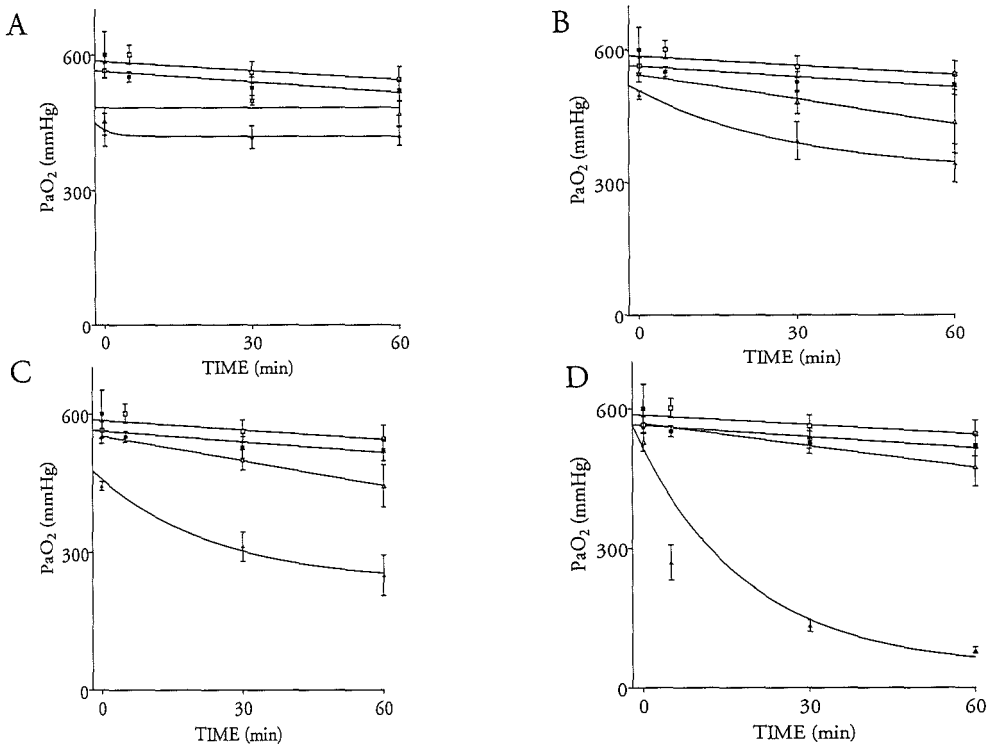

Figure 4. *Effect of different concentrations PG on arterial oxygenation*
Arterial oxygenation was monitored throughout the experiment by drawing arterial blood samples at start of the experiment, immediately after instillation of the fluorescent labeled liposomes, and then after 30 min and 60 min . □, 0% PG; ■ normal; , ▲ liposome suspended in PBS [20% (A), 40%(B), 60% (C), 86%(D)]; Δ liposomes resuspended in solution 2.

surfactant proteins on the uptake of DPPC. Both SP-A and SP-C have been shown to enhance the uptake of liposomes by alveolar cells[2-5, 25, 26]. Besides surfactant proteins and DPPC, also the other individual surfactant lipids can influence the uptake; Quintero et al. and Poelma et al. demonstrated the enhanced uptake of PG containing liposomes by isolated alveolar macrophages and alveolar type II cells[6, 7]. However, these studies were performed *in vitro* and significant differences between *in vivo* and *in vitro* experiments have been reported[15].
Since surfactant therapy is nowadays more common in clinical practice, and studies on surfactant therapy mostly focus on the addition of surfactant proteins[27-29], very little information is available with regard to the influences of the individual surfactant lipids on uptake or on lung function.
Therefore, the current study investigated the effects of the second major component of surfactant, PG, on the uptake in ventilated rats, as well as the effects on endogenous surfactant function by monitoring arterial oxygenation and determining lung mechanics (protein in lavages fluid, Gruenwald index). The study was performed in healthy animals, as the normal function/role of PG has to be clarified to provide a basis for changes possibly found in diseased animals.

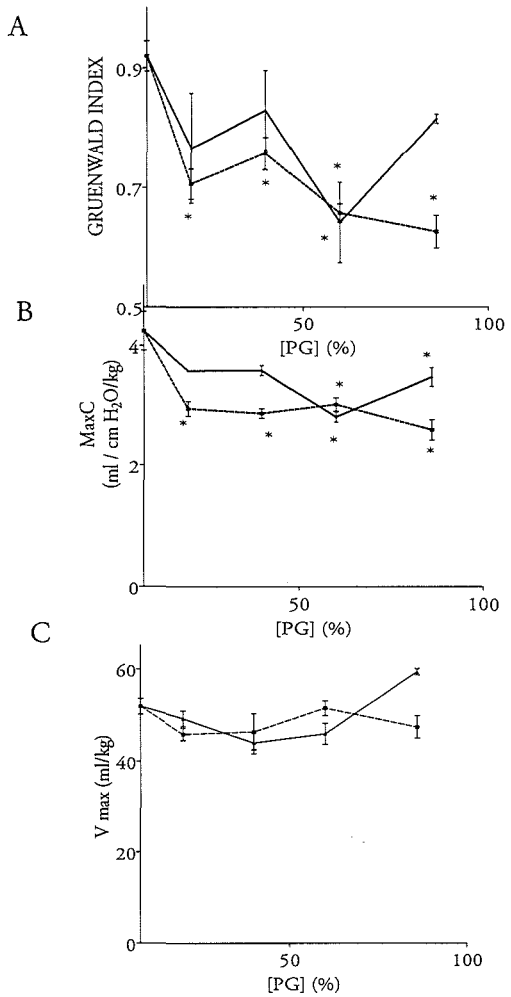

Figure 5. *Effect of different concentrations PG on lung mechanics*
One hour after instillation of the fluorescent labeled liposomes suspended either in PBS (dotted line) or in solution 2 (solid line), the rats were sacrificed and static pressure-volume curves were assessed. As a measure of lung mechanics, the Gruenwald index (A), maximal compliance (MaxC; B) and maximal volume at 35 cm H_2O (V max; C) were determined (n=4 rats at each concentration; values are mean ± SEM). * indicates a significant difference ($p < 0.05$) in comparison to the normal (8% PG) liposomes.

As demonstrated by the results of the current study, incorporation of PG does influence the uptake of surfactant-like liposomes by alveolar cells, although the effects on both cell types differ. Only at a 20% PG concentration is a significant decrease in the uptake of liposomes by alveolar type II cells seen, whereas at the other PG concentrations no effects were observed. For alveolar macrophages the influence of intratracheal instillation of PG containing liposomes is more dramatic; in particular, on the number of alveolar macrophages obtained from the BAL after instillation of PG containing liposomes. Even the incorporation of 20% PG, which is approximately twice that of the normal PG concentration (8%), within the liposomes resulted in a decreased number of macrophages collected from the BAL. Histological samples of the lungs of animals that were instilled with liposomes containing 86% PG revealed complete absence of alveolar macrophages in the lung. The cells might have migrated to bronchial associated lymphatic tissue or elsewhere, or perhaps the cells did not leave but were killed by overstimulation. From the current results, although it cannot be excluded that the alveolar macrophages migrated elsewhere, we hypothesize that they were overstimulated and killed in

the process. On the other hand, *in vitro* an increase in the concentration PG does not induce a decrease of alveolar macrophages[7]; nevertheless, it has to noted that the incubation of the alveolar macrophages and labelled liposomes takes place in solution 2, and thus 'essential' cofactors are already present.

The relation between the decrease/absence of alveolar macrophages in the alveolar lavages fluid and the deterioration in surfactant function, i.e. a worsening arterial oxygenation, increased amounts of proteins in the BAL and a decreased Gruenwald index, might be explained by at least two options: First, the presence of a variety of cytokines, inflammatory mediators and proteolytic enzymes[30] produced by stimulated alveolar macrophages of which some have been suggested to be related to respiratory failure[31-35] might relate the absence of alveolar macrophages to the deteriorated surfactant function. Next, the increased PG content of the liposomes leads to overstimulation of the alveolar macrophage and consequently bursting of the macrophage, releasing its contents into the alveolar space. However, after this expulsion of the contents of macrophages has taken place, these substances are removed very rapidly and can hardly be measured. However, damage to the surfactant monolayer is already made and a vicious circle is initiated. Protein concentration will rise in the alveolar space and result in a dose-dependent inactivation/inhibition of surfactant[36]: surfactant is rate-limiting for the transfer of protein over the alveolar capillary membrane, loss of surfactant function will result in more protein influx, which in turn will inactivate/inhibit even more surfactant[36-38]. Further evidence that the surfactant system is damaged and that there is no additional fibrosis present comes from the Vmax data, which is similar in both groups (Fig. 5C). This demonstrates that when sufficiently high pressures are applied, collapsed alveoli can still be recruited.

Despite these severe effects of PG on the surfactant metabolism, more interestingly the adverse consequences of increasing amounts of PG can be avoided. When the liposomes are suspended in solution 2, rather than in PBS, the amount of alveolar macrophages is not decreased in contrast to the similar liposomes resuspended in PBS, except for liposomes containing 60% PG. In addition, when the amount of PG is increased and the liposomes are resuspended in solution 2, a significant increase in uptake by alveolar macrophages is observed, showing a maximum uptake at a PG concentration of 20%. This increased uptake of liposomes with a high content of PG incorporated and suspended in solution 2 are similar to the results obtained by Poelma et al. with regard to the influence of PG on the uptake of liposomes, suspended in solution 2, by alveolar macrophages in vitro[7]. In the present study, resuspending the liposomes in solution 2 has hardly no influence on the uptake of these liposomes by alveolar type II cells, whereas in vitro the uptake of liposomes by alveolar type II cells is stimulated by the incorporation of PG in these liposomes suspended in solution 2. Although both studies were performed using the same technique, the effects of a high PG concentration on the uptake by alveolar type II cells is different. It can thus be concluded that, besides the presence of the substances in solution 2, in both the current study and the previously one, additional environmental factors affect the uptake of surfactant-like liposomes by alveolar type II cells *in vivo*.

The protection obtained by suspending the liposomes in solution 2 may be explained in several ways. Obviously, the presence of divalent cations (Mg, Ca) in solution 2 might 'neutralize' the negative charge of PG. Another explanation could be that the presence of components necessary for alveolar macrophages and normal lung function in solution 2 (e.g. Ca, Mg and glucose), replenishes the dilution of these factors in vivo caused by the intratracheal instillation of 1 ml liposome suspension. Since the volume of the liquid phase is estimated to be approximately 200 µl in the rat, instillation of 1 ml will lead to a six-fold dilution of the intra alveolar solutes.

In summary, the present study shows that varying the PG concentration in intratracheally instilled liposomes may have serious consequences for alveolar macrophages as well as normal lung functioning. A practical implication may be that not only the presence of the hydro-

phobic surfactant proteins are important for surfactant therapy, but also the PG concentration in the applied surfactant may play an important role in surfactant therapy. Therefore, further studies are needed to elucidated the complex mechanism(s) that underlie the results described above not only to improve our knowledge of the surfactant homeostasis but also to achieve improved therapeutic interventions.

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CHAPTER 5

Distinct effects of SP-B and SP-C on the uptake of surfactant-like liposomes by alveolar cells *in vivo* and *in vitro*

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Abstract

The effects of SP-B and SP-C on the uptake of surfactant like liposomes by alveolar type II cells and alveolar macrophages were studied both *in vivo* and *in vitro*. *In vivo*, mechanically ventilated rats were intra tracheally instilled with fluorescent labeled liposomes that had SP-B and/or SP-C incorporated in different concentrations. Consequently the alveolar cells were isolated and cell-associated fluorescence was determined using flow cytometry. The results show that the incorporation of SP-B does not influence the uptake, nor in the presence of essential co-factors. The inclusion of SP-C in the liposomes enhanced the by alveolar type II cells at a SP-C to lipid ratio of 2:100. If divalent cations (calcium and magnesium) were present at physiological concentrations in the liposomes suspension, uptake of liposomes by alveolar macrophages was also enhanced. *In vitro*, the incorporation of SP-B affected uptake only at a protein to lipid ratio of 8:100, whereas the inclusion of SP-C in the liposomes leads to an increased uptake at a protein to lipid ratio of 1:100. From these results it can be concluded that SP-B is unlikely to affect uptake of surfactant, whereas SP-C in combination with divalent cations and other solutes are capable of increasing the uptake.

Introduction

Pulmonary surfactant is a complex mixture of proteins and phospholipids synthesized and secreted by alveolar type II cells. Alveolar surfactant is metabolized and removed from the lung by reuptake of inactive surfactant, by both alveolar macrophages and alveolar type II cells. At least four proteins are known to exist in endogenous surfactant, surfactant protein A (SP-A), SP-B, SP-C and SP-D. From these surfactant specific proteins, SP-B and SP-C, two small hydrophobic proteins, have been demonstrated to play a role in the formation of a stable surfactant monolayer. Especially SP-B has been shown to be essential for normal surfactant function, lowering surface tension[1, 2], as the absence of SP-B at birth leads to death caused by respiratory insufficiency[3, 4] and conditional knockout of SP-B in adult animals leads to respiratory failure[5]. In addition, previous studies have demonstrated altered concentrations of SP-B in broncho-alveolar lavages fluids of patients suffering ARDS[6, 7].

SP-C also enhances surface active properties of surfactant[1, 2, 8-10], although absence of SP-C at birth is not lethal like SP-B, but experiments performed in specific SP-C knock-out mice demonstrated that despite the fact that surfactant pool sizes and lung morphology were similar in wild type and SP-C-knock-out mice, the absence of SP-C lead to a decreased stability of the surfactant at low volumes[11]. Another function of SP-C is increasing the resistance of surfactant against inactivation by plasma proteins[12].

Nowadays, exogenous surfactant is used in clinical practice in neonates, while the use of exogenous surfactant in adults is under consideration[13]. These exogenous surfactant preparations commonly contain SP-B and/or SP-C as these proteins play an important role in lowering surface tension. Though the beneficial effects of addition of the proteins on surface tension lowering activity will not be a point of discussion, their effects on the uptake of surfactant have to be clarified. Uptake of surfactant is an important step in the recycling of surfactant, which is known to be crucial in the maintenance of surfactant homeostasis.

Though both these hydrophobic surfactant proteins have been demonstrated to participate in the re-uptake of inactive surfactant[14, 15] by alveolar type II cells, these studies were done in *in vitro*. Recently we were able to show the influence of the local environment on the uptake of surfactant by alveolar cells[16], indicating the need of studying surfactant metabolism, especially uptake not only *in vitro* but also *in vivo*.

Therefore we examined, the effect of SP-B and SP-C on the uptake of lipids by both alveolar

type II cells and alveolar macrophages *in vivo* in ventilated rats as well as in vitro using fluorescent labeled liposomes, flow cytometry and confocal laser scanning microscopy.

Materials and methods

Ethical Guidelines
This study was approved by the Institutional Animal Committee at the Erasmus University Rotterdam.

Liposome preparation

Both SP-B and SP-C were purified from lipid extracts of porcine lung lavage to homogeneity by Sephadex LH-60 chromatography (Pharmacia, Uppsala, Sweden)[17]. Surfactant-like liposomes were prepared by mixing the following lipids; dipalmitoyl phosphatidylcholine (DPPC), phosphatidyl choline (PC), phosphatidylglycerol (PG), phosphatidyl inositol (PI), and phosphatidyl ethanolamine (PE) labeled with Rhodamine in the head group (Rhodamine DHPE; Molecular Probes, Leiden, The Netherlands) and cholesterol in a weight ratio of 55:21:8:2:6:8 and the indicated concentrations of surfactant proteins. Subsequently, this mixture was dried under a stream of nitrogen. The lipids were purchased from Sigma, Zwijndrecht, The Netherlands, unless stated otherwise. The liposomes were either suspended in PBS or in solution 2 (140 mM NaCl, 5 mM KCl, 2.5 mM phosphate buffer, 10 mM HEPES, 6 mM glucose, 2.0 mM $CaCl_2$ and 1.3 mM $MgSO_4$) at a concentration of 1 mg lipids/ml using glass pearls and vortexing. Immediately prior to use the liposome suspension was sonicated for 2 min on ice using an ultrasonic disintegrator (Branson Sonifier 250, Danbury, USA) to prepare small unilamellar liposomes[16]. The size of the liposomes was determined by dynamic light scattering at 25°C with a Malvern 4700 system using a 25 mW He-Ne laser (NEC, Tokyo, Japan) and the automeasure version 3.2 software (Malvern Ltd, UK). As a measure of particle size distribution of the dispersion, the system reports a polydispersity index (p.d.). This index ranges from 0.0 for a monodisperse and up to 1.0 for an entirely polydisperse dispersion. After ultrasonification the liposomes size ranged from 140 to 165 nm and the polydispersity index ranged from 0.2 to 0.35.

Intratracheal instillation of fluorescent liposomes
The studies were performed with male Sprague-Dawley rats (IFFA Credo, The Netherlands) with a bodyweight of 318 ± 16 g. After induction of anesthesia with a mixture of nitrous oxide (66%), oxygen (33%) and isoflurane (1-2%) a sterile polyethylene catheter (0.8 mm o.d.) was inserted into one of the carotid arteries. The animals were then tracheotomized and a sterile metal cannula was inserted into the trachea.
After these surgical procedures, gaseous anesthesia was ended and replaced with an intraperitoneal injection of pentobarbital sodium (60 mg/ml, Nembutal®, Algin BV, Maassluis, The Netherlands) at a dose of 30 mg/kg bodyweight every hour.
Muscle relaxation was induced and maintained by an hourly intramuscular injection of pancuronium bromide (2 mg/kg, Pavulon; Organon Technika, Boxtel, The Netherlands). The animals were then mechanically ventilated with a Servo® ventilator 900C (Siemens-Elema, Solna, Sweden) set to pressure control mode using a frequency of 30/min, an inspiratory/expiratory ratio of 1:2, a positive end-expiratory pressure (PEEP) of 2 cm H_2O, a peak inspiratory pressure (PIP) of 12 cm H_2O and FiO_2 was set to 1.
Before instillation of the labeled liposomes, PEEP was increased to 6 cm H_2O and PIP was increased to 26 cm H_2O. After disconnection from the ventilator, the liposomes were administered intratracheally at the indicated dosage. The suspension of liposomes (1 mg

lipids/ml, unless stated otherwise) was administered as a bolus of 3 ml/kg followed by a bolus of air (12 ml/kg), directly into the endothracheal tube via a syringe, and the animals were immediately reconnected to the ventilator. Thirty min after instillation of the liposomes PEEP was reduced to 2 cm H_2O and PIP to 12 cm H_2O.
Arterial blood gas values were measured with conventional methods (ABL 555, Radiometer, Copenhagen, Denmark) at start of ventilation, immediately after instillation of the liposomes and every 30 minutes thereafter. One hour after ventilation the animals were sacrificed by exsanguination via the abdominal aorta and the alveolar cells were isolated to determine the cell-associated fluorescence. Untreated animals were sacrificed immediately after anesthesia and their isolated alveolar type II cells and alveolar macrophages were used to correct for auto-fluorescence.

Isolation of alveolar type II cells and alveolar macrophages
Prior to isolation of the cells, the thorax was opened and the blood cells were removed from the lungs by perfusing the pulmonary artery with warmed saline (37°C) supplemented with 20 IE heparin per ml (Leo Pharma, Weesp, The Netherlands). The lungs were removed from the thoracic cavity en bloc and lavaged with 10 ml of solution 1 [140 mM NaCl, 5 mM KCl, 2.5 mM $Na_2HPO_4.2H_2O$, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethaesulfonic acid), 6 mM glucose, 0.2 mM EGTA (ethylene glycol-bis- (β-amino ethyl ether) N,N'-tetra-acetic acid), pH 7.40] at 37°C. This procedure was repeated four times. The lung lavages were pooled per animal and centrifuged (100x *g*; 10 min; 4°C). The cellular pellet, i.e. alveolar macrophages, were suspended in solution 2 to a concentration of $2x10^6$ cells/ml and stored on ice until further use. The alveolar type II cells were isolated according to Dobbs et al.[18]; using enzymatic digestion as previously described[16]. Alveolar type II cells were suspended in solution 2 at a concentration of $2x10^6$ cells/ml and stored on ice until further use. Alveolar macrophages were identified using monoclonal antibodies specific for rat macrophages (ED9) and alveolar type II cells were identified using an alkaline phosphatase assay as described by Edelson et al.[19]. The average yield of alveolar type II cells was $16x10^6$ with a purity of 80 ± 5% and $5x10^6$ alveolar macrophages with a purity of 92 ± 5% per rat.

In vitro uptake of SP-B and SP-C containing liposomes
Alveolar cells of untreated animals were isolated as described above and suspended in *solution* 2 to a concentration of $2x10^6$ cells/ml. A total of $3*10^5$ cells were incubated with the indicated concentration of the specified liposomes at 37°C (final volume 500 µl) in a shaking water bath. After 1 hour, the incubation was terminated by addition of 2 ml of ice-cold PBS (4°C). The cell-suspension was centrifuged at 100x *g* for 10min at 4°C. The supernatant was removed, and the cells were suspended in 2 ml ice-cold PBS and centrifuged again. This wash procedure was repeated twice. Finally, the pellet was resuspended in 200 µl of cold PBS, and cell-associated fluorescence was determined as described below.

Flow cytometry
Cell-associated fluorescence of the alveolar type II cells and alveolar macrophages as a measure for internalized liposomes was determined using flow cytometry (FACSCalibur; Becton Dickinson; Mountain View, USA). The cell-associated fluorescence of 15,000 cells was determined. Alveolar macrophages and alveolar type II cells derived from control animals were used in each experiment to determine the auto fluorescence of the cells. Subsequently, the mean cell-associated fluorescence was determined only for those cells that had a higher fluorescence than that caused by auto-fluorescence (gated cells).

Localization of cell-associated fluorescence
To localize the cell-associated fluorescence, intra-cellular or extra-cellular, confocal laser micrographs were obtained using a confocal microscope (LSM510NLO, Carl Zeiss Jena, Germany). Images were created with plan apochromat 63x 1.4 n.a., objectives and photomultiplier tubes and emission filter set at 500-550 nm. (Rhodamine, excitation 543 nm HeNe laser). Images of alveolar cells were serially sectioned pinhole at 1 airy unit with an interval of 0.5 μm to distinguish cell membrane-associated fluorescence from the intracellular fluorescence.

Statistical analysis
Differences between the different groups that received liposomes with a different composition were analyzed using an ANOVA followed by a Tukey post-hoc test. The unpaired *t*-test was used for analysis of the effect of calcium. Blood gas values were analyzed using a repeated measurement ANOVA.
Differences were considered statistically significant at a $p < 0.05$. Values are expressed as mean ± SD.

Results

Effect of surfactant protein B and C on the uptake in vivo
Though SP-B and SP-C have been shown to influence the uptake by alveolar cells[20-22], most studies were performed *in vitro*[21, 22], and due to the methods used no comparison between *in vivo* and *in vitro* results can easily be made. Therefore in the current study mechanically ventilated rats were intratracheally instilled with fluorescent labeled liposomes containing surfactant proteins B and/or C. Since it has been described that divalent cations effect the function of these surfactant proteins, additional groups were included which were instilled with liposomes suspended in solution 2; which includes calcium and magnesium.The inclusion of SP-B and/or SP-C in the liposome does not influence the uptake by alveolar cells when these liposomes are suspended in PBS (Fig. 1A, C). However, suspending the liposomes in solution 2 leads to a significant increase in uptake by alveolar macrophages of liposomes with SP-C or both SP-B and SP-C incorporated. (Fig. 1 C).
The use of flow cytometry to measure the uptake of these fluorescent labeled liposomes also provides the opportunity to measure the percentage of cells involved in the uptake. Hence, the current experiments demonstrate that the incorporation of SP-B and/or SP-C in the liposome does not affect the number of alveolar cells involved in the uptake (Fig 1B, D), though suspending the liposomes in solution 2 results in a significant increase in the percentage alveolar type II cells involved in the uptake of SP-C containing liposomes and the amount of alveolar macrophages taking up liposomes containing both SP-B and SP-C (Fig 1 B, D).

Effect of increasing SP-B/lipid ratio on the uptake in vivo
The absence of any effect with the inclusion of SP-B in the liposomes raised the question if the amount of SP-B was sufficient or not. Therefore the SP-B/lipid ratio in the liposomes was varied. Higher SP-B/lipid ratios however did not affect the amount of liposomes taken up by individual alveolar type II cells nor the number of alveolar type II cells involved in the uptake (Fig 2A, B)
In addition, increasing the SP-B/lipid ratio did not have an effect on the uptake of the liposomes by alveolar macrophages, as cell-associated fluorescence, as a measure for the amount of liposomes internalized, did not differ significantly from standard liposomes (Fig 2C). The number of alveolar macrophages, taking up the labeled liposomes, tends to decrease with higher SP-B/lipid ratios though this decrease is not significant (Fig 2 D).

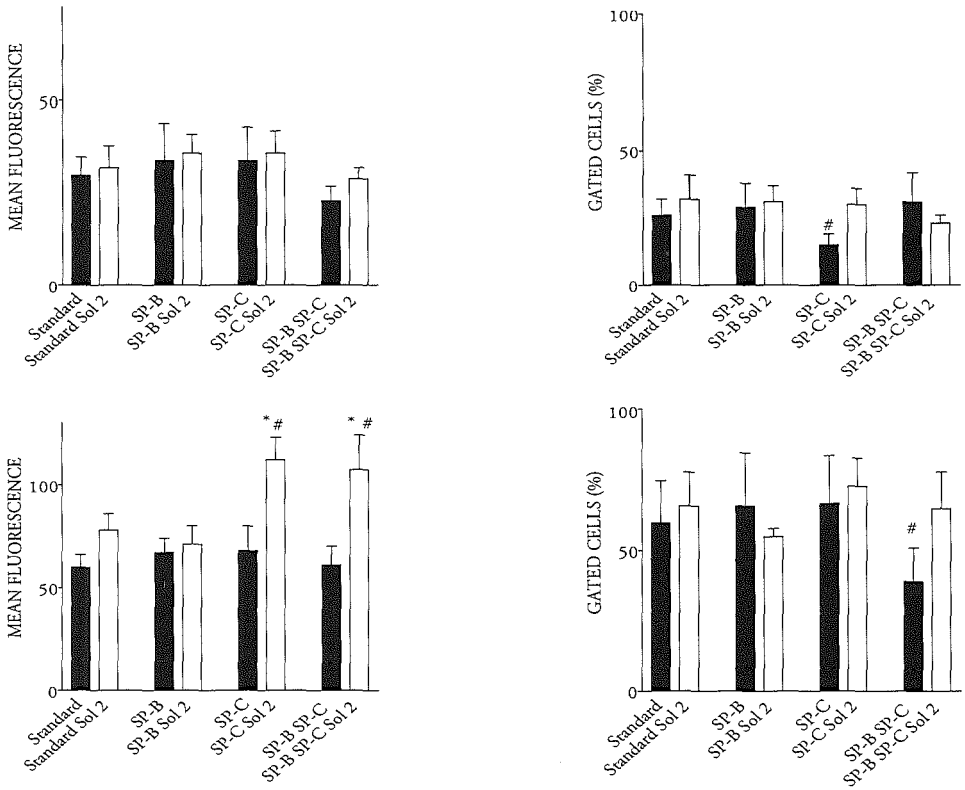

Figure 1. *Effect of SP-B and SP-C on the uptake*
One hour after instillation of the indicated fluorescent liposomes (protein to lipid ratio :100), the alveolar cells were isolated. The mean cell-associated fluorescence of the gated alveolar type II cells (A) and alveolar macrophages (C) was determined. In addition, the percentage gated alveolar type II cells (B) and alveolar macrophages (D) were determined. (n = 6 rats at every concentration; values are mean ± SD). *, $p<0.05$ versus standard, #, $p<0.05$ versus same liposome suspended in PBS.

Resuspending the liposomes in solution 2 did not have any effect on the uptake by either-alveolar type II cells nor by alveolar macrophages (Fig 2).

Effect of SP-C/lipid ratio on the uptake in vivo
To determine the presence of an optimal SP-C/lipid ratio for internalization of these liposomes by alveolar cells, liposomes with different SP-C/lipid ratios were instilled. SP-C has a maximal effect on the uptake by alveolar type II cells at a SP-C /lipid ratio of 2:100 (Fig. 3A). Increasing the SP-C/lipid ratio did not affect the number of alveolar type II cells involved in the uptake (Fig. 3C).
Interestingly, uptake of liposomes by alveolar macrophages is unchanged with increasing SP-C/lipid ratios when these liposomes are resuspended in PBS. However when the liposomes are resuspended in solution 2, significant increases in uptake are demonstrated when SP-C is incorporated in the liposome, with a maximal uptake at a SP-C/lipid ratio of 2:100 (Fig 3C). Though, the number of alveolar macrophages involved in the uptake remains unaffected by

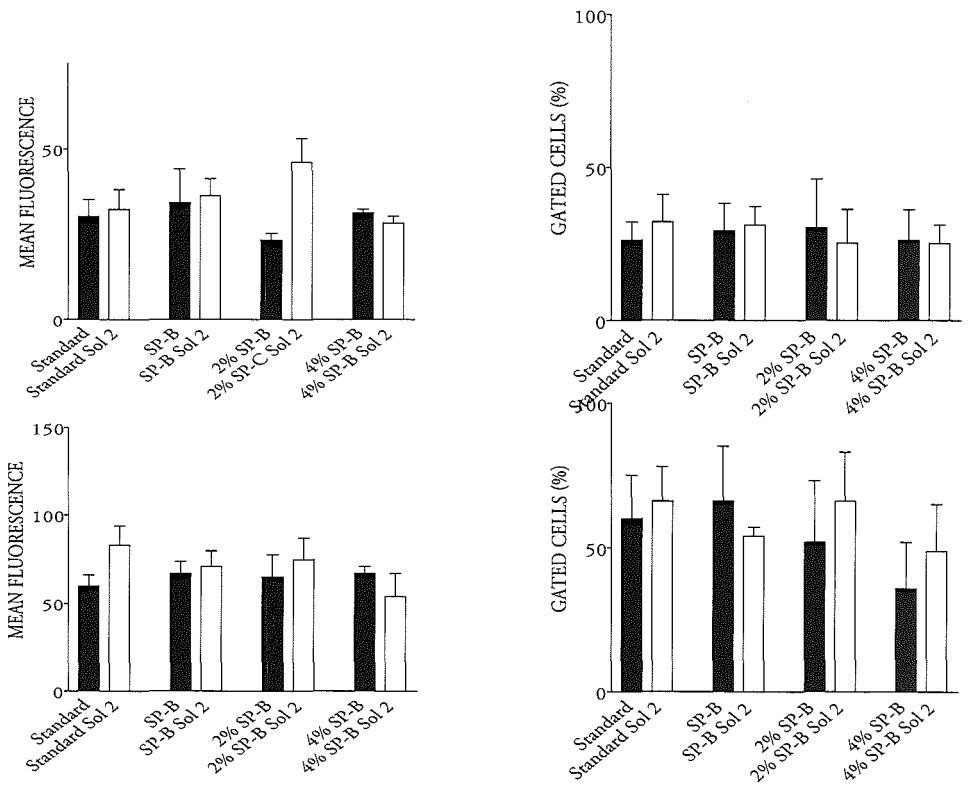

Figure 2. *Effect of increasing the SP-B: lipid ratio's on the uptake*
One hour after instillation of the fluorescent liposomes with indicated concentrations SP-B, the alveolar cells were isolated. The mean cell-associated fluorescence of the gated alveolar type II cells (A) and alveolar macrophages (C) was determined. In addition, the percentage gated alveolar type II cells (B) and alveolar macrophages (D) were determined. (n = 6 rats at every concentration; values are mean ± SD). *, $p<0.05$ versus SP-B. #, $p<0.05$ versus same liposome suspended in PBS.

the incorporation of SP-C in the liposome or resuspension of the liposomes in solution 2 (Fig. 3D).

Effect of SP-B, SP-C containing liposomes on gas exchange

The instillation of the fluorescently labeled liposomes did not have any effect on gas exchange as determined by arterial blood gas values (Table 1). Nor was there any effect on arterial oxygenation by the incorporation of the studied surfactant proteins or suspending the liposomes in solution 2.

In vitro effects on the uptake of liposomes with SP-B or SP-C incorporated

A possible explanation for the absence of any effects of the inclusion of SP-B in the liposomes on the uptake might be that in the healthy lung the amount of SP-B is sufficient or optimal and additional SP-B, added by instillation of liposomes with SP-B incorporated does not affect uptake. To study the effects of the inclusion of SP-B in the liposome in the absence of any

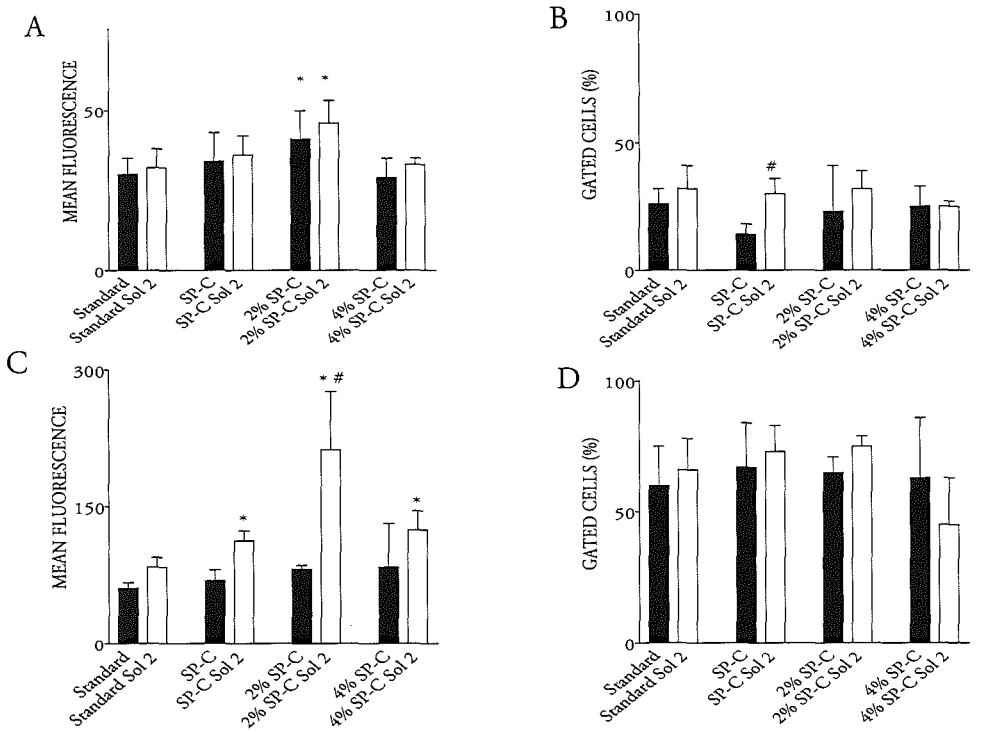

Figure 3. *Effect of increasing the SP-C: lipid ratio's on the uptake*
One hour after instillation of the fluorescent liposomes with indicated concentrations SP-C, the alveolar cells were isolated. The mean cell-associated fluorescence of the gated alveolar type II cells (A) and alveolar macrophages (C) was determined. In addition, the percentage gated alveolar type II cells (B) and alveolar macrophages (D) were determined. (n = 6 rats at every concentration; values are mean ± SD). *, $p<0.05$ versus SP-C; #, $p<0.05$ versus same liposome suspended in PBS.

Table 1. *Arterial oxygenation*

	Start	AL	5'	30'	60'
Standard	540 +/- 33	523 +/- 36	551 +/- 40	522 +/- 28	501 +/- 41

Values are means ± SE. Bloodgas values of animals intratracheally instilled with normal liposomes (n = 5 rats).

natural SP-B, alveolar cells of untreated animals were isolated and $3*10^5$ cells were incubated with different concentrations liposomes in the presence of solution 2. These liposomes had SP-B incorporated in a SP-lipid ratio of 1:100. Uptake of these liposomes was unchanged in comparison to liposomes without SP-B. Neither uptake per cell (Fig 4 A,C) or the number of cells involved in the uptake (Fig 4 B, D) was affected by the incorporation of SP-B in the liposomes.

For SP-C, liposomes with incorporated SP-C in a protein/lipid ratio of 1:100, were added at different concentrations to isolated alveolar cells and incubated for one hour. At the highest used liposomes concentration (50 μg/ml) inclusion of SP-C results in an approximately 4-fold increase in uptake by alveolar type II cells (Fig. 5A). In addition, the incorporation of SP-C in the liposome not only increased uptake of these liposomes by alveolar type II cells, but induced also a significantly higher uptake of these liposomes by alveolar macrophages.
Furthermore, the incorporation of SP-C does not affect the number of cells involved in the uptake at an SP-C/lipid ratio of 1:100 (Fig 5B, D).

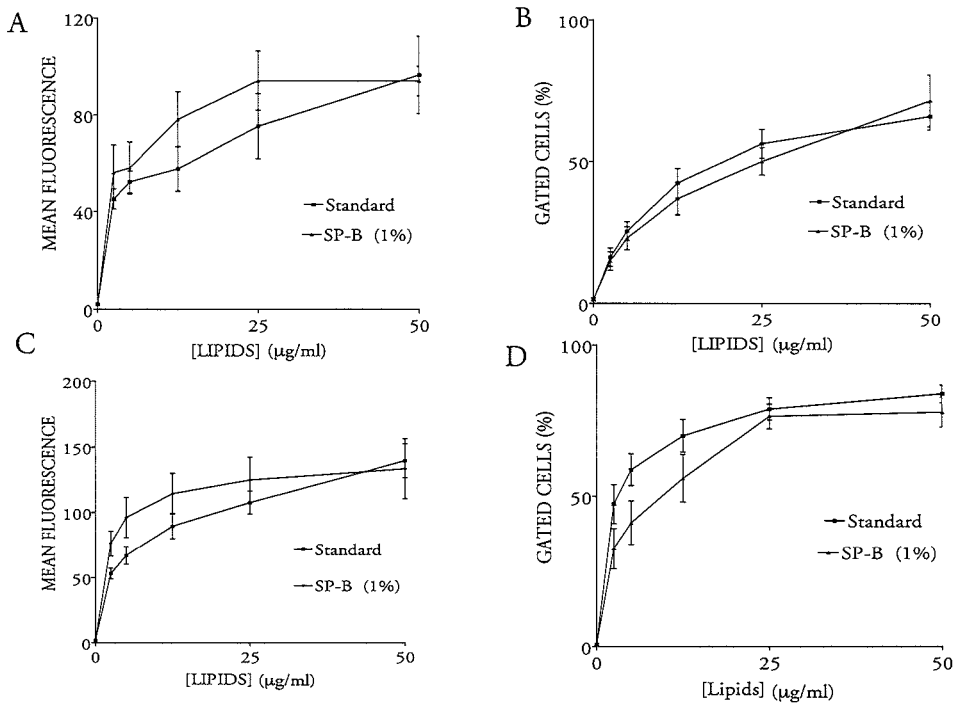

Figure 4. *Concentration-dependent uptake of liposomes with SP-B in vitro*
Alveolar cells were isolated from control animals and incubated for 1 hour at 37°C with the indicated concentrations of fluorescent liposomes dependent on the group containing SP-B or no surfactant proteins. The mean cell-associated fluorescence of gated alveolar type II cells (A) and alveolar macrophages (C) was determined. In addition, the percentage gated alveolar type II cells (B) and alveolar macrophages (D) were determined. No significant differences were observed at any concentration (n = 4 incubations at every concentration; values are mean ± SEM).

Localization of cell-associated fluorescence
To ascertain that the cell-associated fluorescence was located within the cell rather than binding to the outer membrane of the cell, confocal laser scanning microscopy was used. One hour after intratracheal instillation of the liposomes containing SP-B, SP-C in mechanically ventilated rats, the alveolar cells were isolated and confocal scans were made. These confocal scans through the middle of the cell show a punctuate fluorescence throughout the cell limited

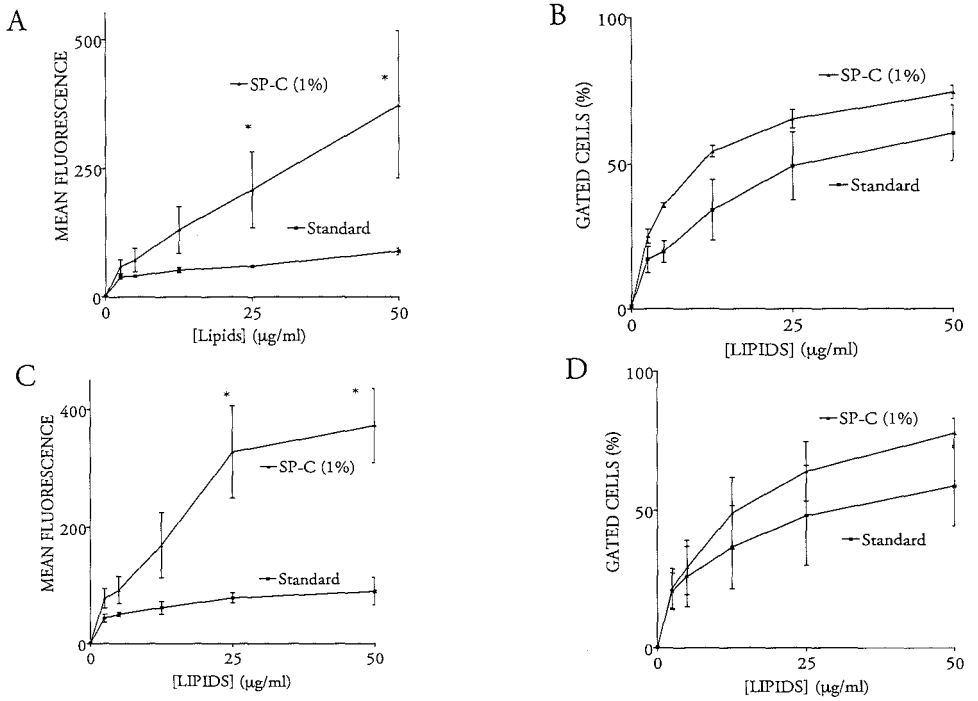

Figure 5. *Concentration-dependent uptake of liposomes with SP-C in vitro*
Alveolar cells were isolated from control animals and incubated for 1 hour at 37°C with the indicated concentrations of fluorescent liposomes dependent on the group containing SP-C or no surfactant proteins. The mean cell-associated fluorescence of gated alveolar type II cells (A) and alveolar macrophages (C) was determined. In addition, the percentage gated alveolar type II cells (B) and alveolar macrophages (D) were determined. (n = 4 incubations at every concentration; values are mean ± SEM). *, $p<0.05$ versus standard.

to its circumference with the exception of its nucleus, demonstrating the intracellular presence of the fluorescent liposomes. By scanning the cells at different levels each 1 µm apart it was ensured that the used confocal scan was indeed through the middle of the cell, in addition, light microscopy of the same cell at the same setting, demonstrated the limitation of the cell-associated fluorescence to the cytoplasm (Fig. 6).

Effect of SP-B/lipid ratio on the uptake in vitro
Though in the current study no effect of inclusion of SP-B in the liposomes on the uptake by alveolar cells was observed, Rice et al. have demonstrated a significant increase in uptake of liposomes by alveolar type II cells with SP-B incorporated[14]. However in their study higher protein/lipid ratios were used. Therefore using our method of fluorescent labeled liposomes, isolated alveolar cells of untreated animals were incubated with 50 µg/ml liposomes that had an increasing amount of SP-B included, varying from 1:100 to 8:100. Increasing the SP-B/lipid ratio results in a significantly enhanced uptake of liposomes by alveolar type II cells at an SP-B: lipid ratio of 8:100 (Fig. 7A). In addition, the inclusion of SP-B in the liposome at

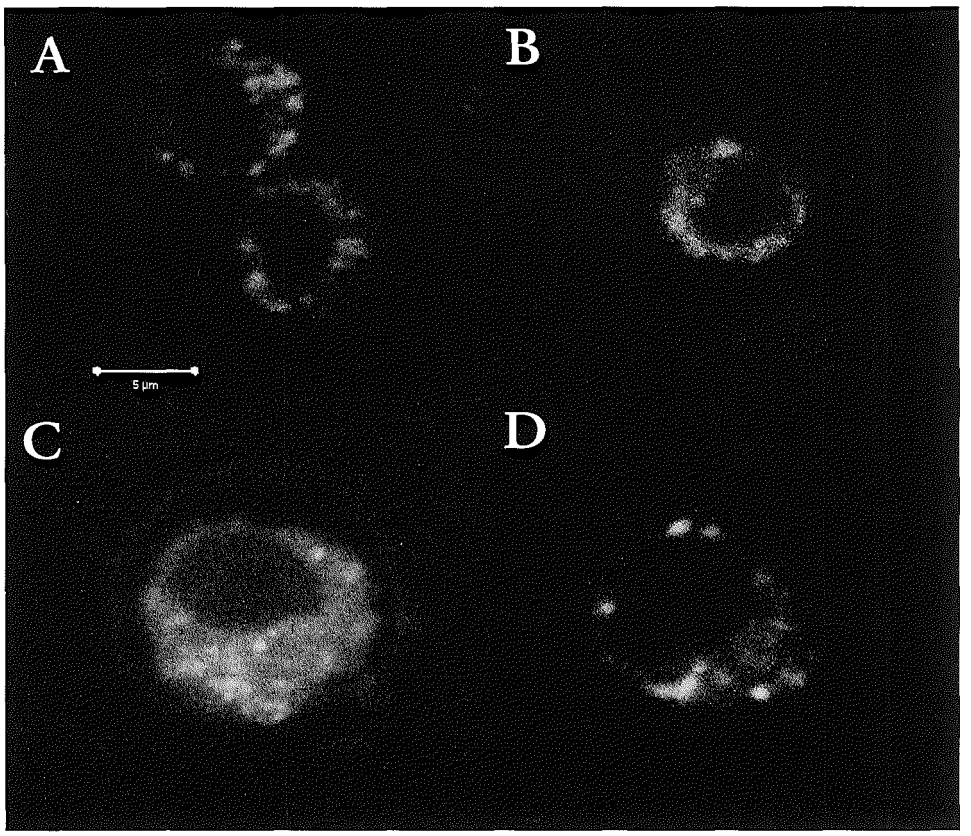

Figure 6. *Localization of cell-associated fluorescence*
Mechanically ventilated rats were intratracheally instilled with fluorescent labeled liposomes containing either SP-B (A, C) or SP-C (B, D). One hour after instillation alveolar macrophages (C, D) and alveolar type II cells (A, B) were isolated and confocal scans were made. All scans were made using the same microscopic settings.

this particular ratio resulted in an increased number of alveolar type II cells involved in the uptake (Fig. 7B)
The effect of an increased SP-B/lipid ratio on the uptake of liposomes by alveolar macrophages demonstrated a similar significant increase at a SP-B/lipid ratio of 8:100. An increased ratio however did not influence the number of alveolar macrophages involved in the uptake of these liposomes.

Discussion

In the current study we investigated the effects of both SP-B and SP-C on the uptake of surfactant like liposomes *in vivo* and *in vitro*.
As demonstrated in this study, SP-B does not influence the uptake of liposomes *in vivo*, not even at a SP-B/lipid ratio of 4:100. However, these liposomes were suspended in PBS prior to

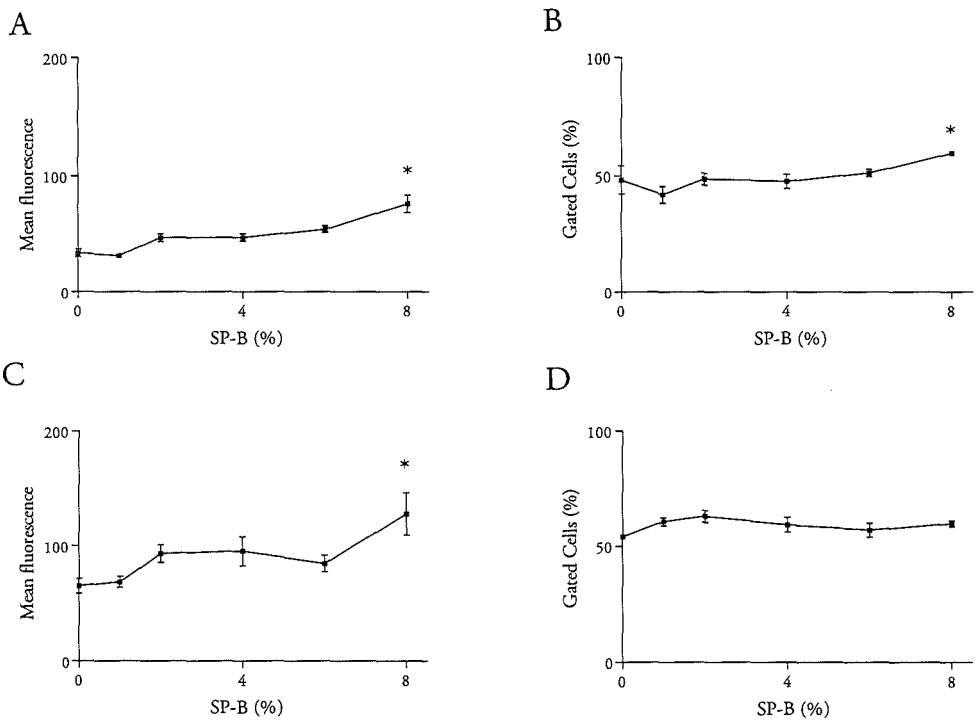

Figure 7. *Effect of increased SP-B: lipid ratio's on the uptake in vitro*
Isolated alveolar cells of untreated animals were incubated with 25 µg/ml of liposomes with a different SP-B/liposome ratio, varying from 1:100 to 8:100. Both alveolar macrophages (C, D) and alveolar type II cells (A, B) were analyzed and mean cell associated fluorescence (A, C) and percentage gated cells (B, D) were determined (n = 4 incubations at every concentration; values are mean ± SEM) *, $p<0.05$

instillation in trachea of the rats. Since the intra-alveolar fluid phase in rats is estimated to be around 200 µl, and the liposomes instilled were suspended in 1 ml PBS, this might lead to dilution of the intra-alveolar pool of solutes in the lung including calcium.
Previous studies have demonstrated a crucial role of calcium on the biophysical function of SP-B as well in interaction with PG or formation of tubular myeline[23, 24]. Therefore, to ensure more physiological circumstances the liposomes were resuspended in solution 2 containing calcium, magnesium, potassium and glucose in similar concentrations as the endogenous pool. However, despite resuspending the liposomes in solution 2, the incorporation of SP-B in the liposome did not affect the uptake of lipids by alveolar cells.
Another explanation might be that the physiological amount of SP-B present *in vivo* is the optimal concentration for the SP-B mediated lipid uptake by alveolar cells, in other words, raising the SP-B concentration by using SP-B incorporated liposomes does not further influence the uptake. Therefore, the uptake of liposomes by isolated alveolar cells was studied *in vitro* using liposomes with and without incorporated SP-B. These experiments clearly demonstrate that at the physiological SP-B/lipid ratio of 1:100 no effect on the uptake of liposomes by alveolar type II cells was observed compared to the uptake of liposomes devoid of SP-B. However, Rice et al. demonstrated a significant increase in uptake of liposomes by

alveolar type II cells in vitro due to the presence of SP-B[14], though at higher SP-B:lipid ratio's. To determine if these results could be repeated and extended to alveolar macrophages using our method, we isolated alveolar cells and incubated these cells with a fixed concentration of lipids while increasing SP-B/lipid ratio up to 8:100. At the highest ratio, a significant increase in uptake was observed indicating that SP-B is indeed able to increase uptake, in accordance with the results of the previous studies[14, 15].

Uptake of liposomes is suggested to take place via a receptor mediated pathway[25-28] though not exclusively; where these "phospholipid" receptors tend to have a higher affinity for negatively charged lipids in comparison to neutrally charged lipids[28]. However the increase in uptake due to the incorporation of SP-B, though in a high protein/lipid ratio (8:100), seems unlikely to be caused by the presence of a specific SP-B receptor. The relatively high concentrations of SP-B necessary to induce an effect on the uptake, make a specific receptor physiologically unlikely or suggest a receptor with a very low affinity. Summarizing, the SP-B containing liposomes are internalized by the "phospholipid receptors, and not by additional SP-B receptors on the alveolar cells. The increment in uptake caused by SP-B at the high protein to lipid ratio could also be explained by changes in lipid conformation of the liposomes, because SP-B is able to change lipid conformation of surfactant[29].

In vivo alveolar type II cells internalize more liposomes when SP-C is incorporated in the liposomes at a protein to lipid ratio of 2:100. At other protein to lipid ratio's no effect of SP-C was detected even when the liposomes were suspended in solution 2. In contrast, significant stimulation of the uptake of liposomes containing SP-C by alveolar macrophages were observed at various lipid to protein ratio's when these liposomes were suspended in solution 2. To study the influence of possible additional effects of 'environmental' factors *in vivo*, the uptake of liposomes with SP-C incorporated (SP-C/lipid ratio of 1:100) was studied *in vitro*. At this ratio, SP-C enhanced the uptake; at a lipid concentration of 50 μg/ml the uptake of SP-C containing liposomes was approximately 6 times greater by both alveolar macrophages and alveolar type II cells. These results suggest that *in vivo* the uptake of SP-C containing liposomes by alveolar cells may be suppressed by some unknown factor(s).

How the incorporation of SP-C in liposomes may enhance the uptake by alveolar cells? Rice et al and Horowitz et al[14, 15] have demonstrated previously that SP-C does enhance the uptake of lipids in a non-saturable way. In addition, SP-C is known to associate very rapidly with lung tissue and alveolar macrophages[30, 31]. This increased association, even more rapidly than DPPC, might be a possible explanation for an increased uptake of liposomes containing SP-C. Although other factors such as the conformation changes observed in liposomes after incorporation of SP-C may also affect the binding and uptake of these liposomes by alveolar cells as was suggested by Rice et al (14).

In summary, this study indicates that SP-B has no effect on the uptake of liposomes in vivo by alveolar cells. However, SP-C was shown to enhance the uptake of liposomes *in vivo* by alveolar cells, an effect which could be further increased by suspending the liposomes in a buffer which contained calcium, magnesium and other solutes which are necessary for normal alveolar function. Probably, not only these solutes may effect the uptake of the 'SP-C-mediated uptake' of liposomes, but also other factors. Since we were able to demonstrate *in vitro* in the presence of physiological concentrations of calcium and magnesium that the 'SP-C-mediated uptake' of liposomes is much higher by alveolar cells than *in vivo*, suggesting that in vivo the uptake appears to be down-regulated. The precise mechanism of this possible down-regulation of the uptake is not known but may include cell-to cell contact and unknown soluble factors.

The results of the current study, absence of effects of SP-B and increased uptake due to SP-C, might be useful in clinical practice; nowadays, more and more exogenous surfactant preparations contain surfactant proteins B and C, as these proteins have been demonstrated to increase efficiency of the surfactant[32].

To date it is widely accepted that small 'inactive' surfactant aggregates are removed from the alveolar space by alveolar cells[33, 34]. Exogenous surfactant is surface active and consists mainly of large 'active' surfactant aggregates. However, after various breathing cycles the exogenous surfactant will become small inactive surfactant aggregates and need to be recycled. The presence of SP-B in these aggregates will probably not influence the uptake of these liposomes by alveolar type II cells, the first step in the recyling. On the other hand, inclusion of SP-C in exogenous surfactant in either the absence or presence of calcium and other solutes may even enhance the recycling of the surfactant by increasing the uptake of surfactant by the alveolar cells. Therefore, variations in the SP-B concentrations of exogenous surfactant will probably have little influence on the recyling of surfactant though variations in the SP-C concentrations may stimulate the recyling. However, it is nowadays unknown whether situation is beneficial for the therapy of respiratory failure, an enhanced recycling or not.

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CHAPTER 6

Exogenous surfactant HL-10 influences uptake of surfactant lipids *in vivo* and *in vitro*

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Submitted

Abstract

Using flow cytometry, the effect of exogenous surfactant on the uptake of fluorescent labeled surfactant-like liposomes was studied *in vivo* and *in vitro*. Healthy adult rats were intratracheally instilled with exogenous surfactant (non-sonicated active or sonicated, inactive) and labeled liposomes. Cell-associated fluorescence of the alveolar type II cells and alveolar macrophages was compared with those derived from animals that received only labeled liposomes. *In vitro*, alveolar cells were incubated with labeled liposomes and sonicated or non sonicated surfactant, or with large or small aggregates, or no surfactant at all.
In vitro, uptake of labeled liposomes by alveolar cells was increased by large, active surfactant aggregates. *In vivo*, both sonicated and nonsonicated surfactant decreased uptake of labeled liposomes by decreasing the percentage cells involved in the uptake in healthy lungs. These results suggest a feedback mechanism; recycling is decreased due to a decrease in uptake, because healthy lungs do not need additional surfactant. This feedback mechanism is likely to be absent *in vitro* because environmental factors (e.g. hormonal inflammatory or nerval parameters) are not present, which might explain the differences between *in vivo* and *in vitro* effect of exogenous surfactant on the uptake.

Introduction

Surfactant metabolism is a complex mechanism of production, secretion, uptake and recycling needed to maintain normal lung function. Disturbances in this complex system are associated with diseases such as neonatal RDS and adult ARDS[1-4]. Exogenous surfactant therapy has become widely accepted in the treatment of RDS and is even under consideration for the treatment of ARDS[5]. Nevertheless, the effects of the administration of exogenous surfactant on the endogenous surfactant metabolism are still under discussion, because there are conflicting results on the influence of large amounts of exogenous surfactant[6-10]. The uptake of inactive surfactant is known to be essential in the surfactant metabolism: especially in neonates rely on recycling for >90%, indicating the need for uptake of inactivated surfactant[11]. In contrast, in adult rabbits only 50% of the endogenous alveolar surfactant is produced by recyling[12].
However, most studies have focused on the production or secretion of endogenous surfactant and little is known on the effect on the uptake. Therefore the current study investigates the effects of exogenous surfactant on the uptake of labeled surfactant-like liposomes by alveolar cells both *in vivo* and *in vitro* in healthy adult rats.

Materials and methods

Ethical Guidelines
This study was approved by the Institutional Animal Committee at the Erasmus MC Rotterdam.

Liposome preparation
Small-unilamellar fluorescent labeled surfactant-like liposomes, containing dipalmitoyl phosphatidylcholine (DPPC), phosphatidyl choline (PC), phosphatidylglycerol (PG), phosphatidyl inositol (PI), and phosphatidyl ethanolamine (PE) labeled in the head group with rhodamine (Rhodamine DHPE; Molecular Probes, Leiden, The Netherlands) and cholesterol in a weight ratio of 55:21:8:2:6:8 were prepared as previously described[13].

Preparation of exogenous surfactant
Exogenous natural surfactant (HL-10, Leo Pharmaceuticals Products, Ballerup, Denmark), isolated as described by Gommers et al.[14], was suspended in warmed saline (37°C) to a concentration of 50 mg/ml for *in vivo* experiments and 25 mg/ml for *in vitro* experiments. *In vivo*, exogenous surfactant was administered at a dosage of 150 mg/kg. Exogenous surfactant was suspended in the liposome suspension resulting in a suspension containing 50 mg/ml exogenous surfactant and 1 mg/ml fluorescent labeled liposomes (liposome to surfactant ratio 1:50).
To study the effect of inactivated surfactant, suspended surfactant was sonicated for 2 min on ice using an ultrasonic disintegrator (Branson Sonifier 250, Danbury, USA) which resulted in a significantly higher surface tension and absence of dynamic properties as determined by Wilhelmy balance as compared to the non-sonicated surfactant (data not shown).

Intratracheal instillation of liposomes
The studies were performed with male Sprague-Dawley rats (IFFA Credo, The Netherlands) with a bodyweight of 314 ± 18 g; the animals were randomly divided in 3 groups. Two groups received, respectively, exogenous surfactant containing fluorescent labeled liposomes (SurfLipo group) or sonicated surfactant containing fluorescent labeled liposomes (SonSurfLipo group). This mixture of exogenous surfactant and labeled liposomes was administered using a technique previously described by Verbrugge et al.[15]. One group of animals did not receive any exogenous surfactant prior to ventilation (Normal group).
The animals underwent surgical procedures and mechanical ventilation as previously described[13]. During mechanical ventilation, arterial blood gases were measured with conventional methods (ABL 555, Radiometer, Copenhagen, Denmark) at start of ventilation, and every 30 min thereafter. The Normal group received fluorescent labeled liposomes immediately at start of ventilation as previously described[13]. One hour after instillation of the fluorescent labeled liposomes, the animals were sacrificed by exsanguination via the abdominal aorta and the alveolar cells were isolated to determine the cell-associated fluorescence. Untreated control animals were sacrificed immediately after anesthesia and their isolated alveolar type II cells and alveolar macrophages were used to correct for auto-fluorescence.

Isolation of alveolar type II cells and alveolar macrophages
Alveolar cells were isolated as previously described using enzymatic digestion using elastase[13]. Prior to isolation of the cells, the thorax was opened and the blood cells were removed from the lungs by perfusing the pulmonary artery with saline (37°C) supplemented with 20 IE heparin per ml (Leo Pharma, Weesp, The Netherlands). The lungs were removed from the thoracic cavity en bloc and lavaged with 10 ml of solution 1 [140 mM NaCl, 5 mM KCl, 2.5 mM $Na_2HPO_4.2H_2O$, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethaesulfonic acid), 6 mM glucose, 0.2 mM EGTA (ethylene glycol-bis- (β-amino ethyl ether) N,N'-tetra-acetic acid), pH 7.40] at 22°C. This procedure was repeated four times. The lung lavages were pooled per animal and centrifuged (100x *g*; 10 min; 4°C). The cellular pellet, i.e. alveolar macrophages, were suspended in solution 2 to a concentration of $2x10^6$ cells/ml and stored on ice until further use. The alveolar type II cells were isolated according to Dobbs et al.[16]. Alveolar type II cells were suspended in solution 2 at a concentration of $2x10^6$ cells/ml and stored on ice until further use. Alveolar macrophages were identified using monoclonal antibodies specific for rat macrophages (ED9) and alveolar type II cells were identified using an alkaline phosphatase assay as described by Edelson et al.[17]. The average yield of alveolar type II cells was $15x10^6$ a purity of 81 ± 6% and $4,8x10^6$ alveolar macrophages with a purity of 90 ± 3% per rat.

In vitro assay on the uptake of fluorescent-labeled liposomes and exogenous surfactant
Alveolar type II cells and alveolar macrophages were isolated from untreated animals as described above and were suspended in solution 2 (140 mM NaCl, 5 mM KCl, 2.5 mM phosphate buffer, 10 mM HEPES, 6 mM glucose, 2.0 mM $CaCl_2$ and 1.3 mM $MgSO_4$) to a concentration of $2x10^6$ cells/ml. A total of $3x10^5$ cells was incubated with 25 µl 0.5 mg/ml fluorescent labeled liposomes and different concentrations of exogenous surfactant (25 mg/ml) resulting in the indicated liposome/surfactant ratio, at 37°C in a shaking water bath. Prior to the incubation, exogenous surfactant was either sonicated for 2 min, or not, as indicated. The total incubation volume was 0.5 ml. After 1 hour, the incubation was terminated by addition of 2 ml of ice-cold PBS. The cell suspension was centrifuged at 100x *g* for 10 min at 4°C. The supernatant was removed and the cells were suspended in 2 ml of ice-cold PBS and centrifuged again. This wash procedure was repeated twice. Finally, the pellet was resuspended in 200 µl cold PBS and cell-associated fluorescence was determined as described below.

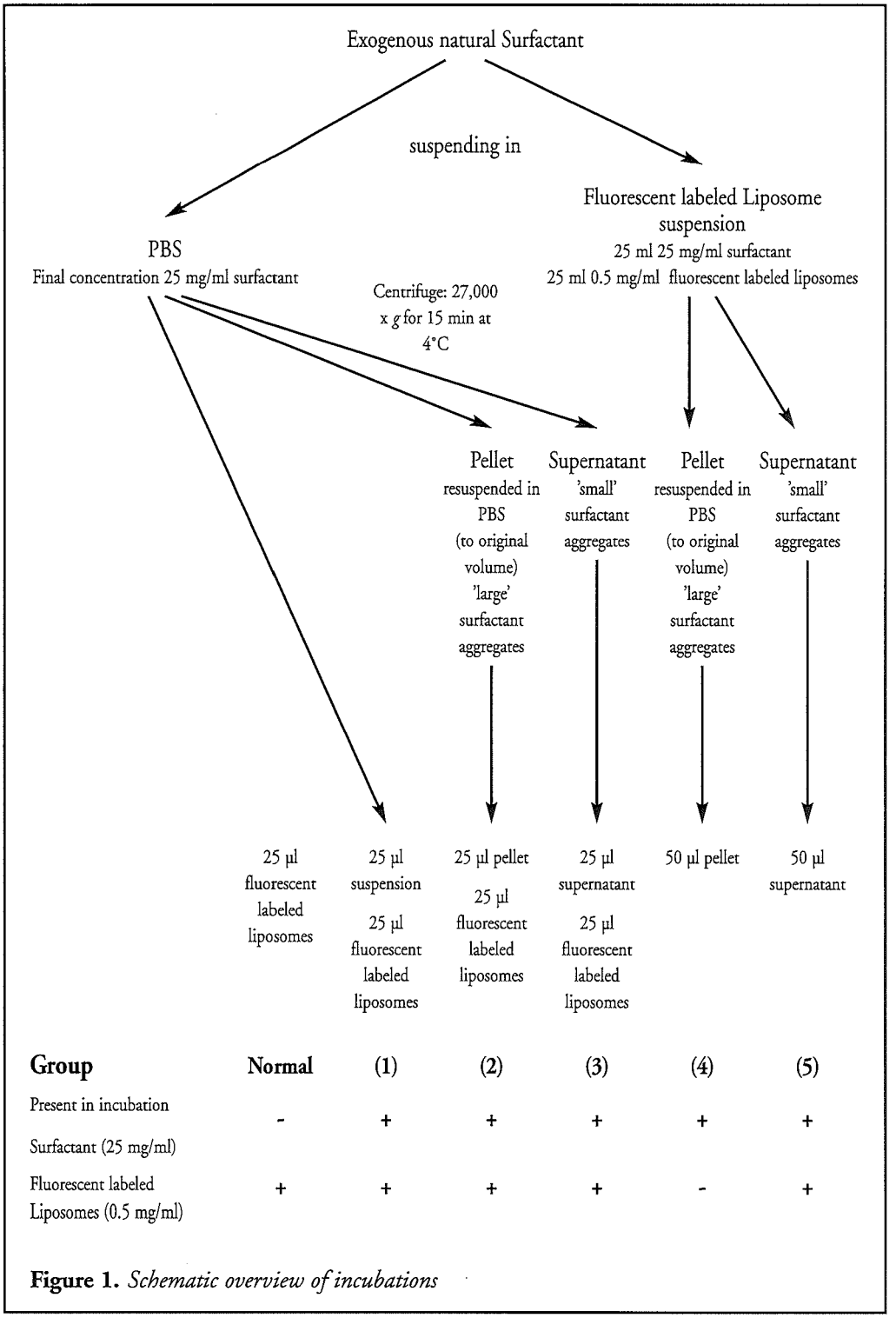
Discrimination of small and large aggregates
To study if the large (pelleted surfactant fraction after centrifugation at 27,000x *g* for 15 min) or small (supernatant of surfactant fraction after centrifugation at 27,000x *g* for 15 min) aggregates within the exogenous surfactant suspension were responsible for a possible effect on the uptake, the following experiment was performed. A total of $3*10^5$ alveolar cells, isolated from untreated animals, were incubated with fluorescent labeled liposomes and exogenous surfactant according to Figure 1. The total volume of the incubation was 500 µl.
In all 6 groups, after 1 hour, the incubation was stopped by adding 2 ml ice-cold PBS. The cell suspension was washed as described above and, finally, the pellet was resuspended in 200 µl cold PBS and cell-associated fluorescence was determined as described below.

Flow cytometry
Cell-associated fluorescence of the alveolar type II cells and alveolar macrophages as a measure for internalized liposomes was determined using flow cytometry (FACSCalibur, Becton Dickinson, Mountain View, CA, USA) as previously described[13].

Localization of cell-associated fluorescence
To inspect the localization of the cell-associated fluorescence confocal micrographs of alveolar cells were obtained using a confocal microscope (Zeiss LSM 510, Carl Zeiss Jena, Germany). Images were created with 63x 1.4 n.a. plan apochromat objective and standard photo-multiplier tubes dedicated to the appropriate excitation and emission spectra of rhodamine (excitation 543 nm and emission 572 nm). Images of alveolar cells were serial sectioned with an interval of 0.5 µm, to distinguish cell-membrane associated fluorescence from true intracellular fluorescence.

Statistical analysis
Differences between different groups was analyzed using an ANOVA followed by a Bonferonni post-hoc test. Differences between the sonicated and non-sonicated surfactant *in vitro* were analyzed using an independent t-test. Differences were considered significant at a $p < 0.05$. Values are expressed as mean ± SEM.

Results
Prior to the experiments surface activity of surfactant suspended in the suspension containing fluorescent labeled liposomes was determined and ensured to be unaffected (data not shown).



Group	**Normal**	(1)	(2)	(3)	(4)	(5)
Present in incubation	-	+	+	+	+	+
Surfactant (25 mg/ml)						
Fluorescent labeled Liposomes (0.5 mg/ml)	+	+	+	+	-	+

Figure 1. *Schematic overview of incubations*

Influence of exogenous surfactant on the uptake in vitro

The influence of exogenous surfactant on the uptake of liposomes *in vitro* was studied by incubating alveolar cells with 25 µg/ml of fluorescent labeled liposomes and increasing concentrations of exogenous surfactant. The addition of exogenous surfactant (non-sonicated) induced an apparent increase in uptake by alveolar type II cells, with a significant maximum at a liposome to surfactant ratio of 1:50 compared to the uptake of liposomes in the absence of surfactant (Fig 2A). Also, the number of alveolar type II cells involved in the uptake was increased at the same liposome to surfactant ratio (Fig. 2B). When the exogenous surfactant is sonicated, the uptake of liposomes by alveolar type II cells, as well as the number of these cells involved in the uptake, is significantly decreased (Fig 2A+2B).

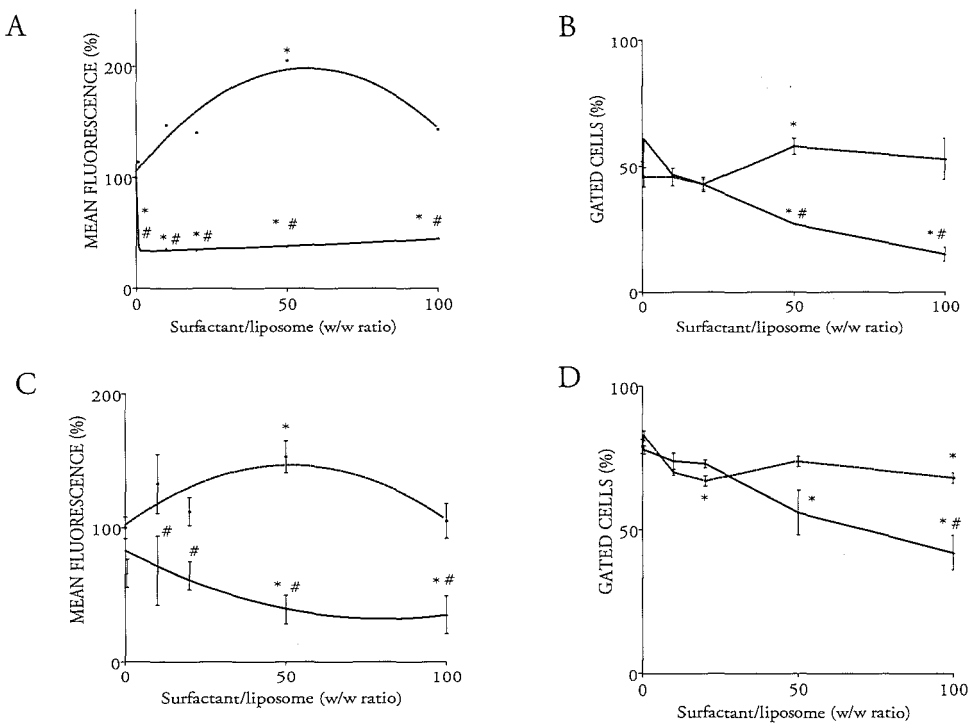


Figure 2. *Effect of active exogenous surfactant on uptake in vitro*

Alveolar type II cells (A, B) and alveolar macrophages (C, D) of untreated animals were isolated and a total of $3*10^5$ cells were incubated with 25 µg/ml fluorescent labeled liposomes for 1 hour at 37°C. Exogenous surfactant, either sonicated inactive (solid line) or non-sonicated active (dotted line), was added to the incubation at the indicated surfactant to liposome ratio. Mean cell-associated fluorescence (A, C) as well as the percentage of cells with a fluorescence higher than auto-fluorescence (B, D) were determined. The cell-associated fluorescence was expressed as the percentage of the cell-associated fluorescence in the absence of exogenous surfactant. Values are expressed as mean ± SEM (n = 4 incubations). * indicates a significant difference compared to the incubation without exogenous surfactant ($p<0.05$). # indicates a significant difference between a similar incubation with non-sonicated surfactant ($p<0.05$).

The addition of exogenous surfactant demonstrated a similar effect on the uptake of liposomes by alveolar macrophages as was observed for alveolar type II cells; again, a significant increase at a liposome to surfactant ratio of 1:50. However, the percentage alveolar macrophages taking up liposomes was significantly decreased at a liposome to surfactant ratio of 1:20 and 1:100, although at a liposome to surfactant ratio of 1:50 no significant difference compared to Normal group (no surfactant) was observed (Fig 2D). Sonification of the exogenous surfactant induced a significant decreae in mean fluorescence and number of alveolar cells participating in the uptake starting at surfactant ratio of 1:50 and higher (Fig 2D).

Effect of large and small surfactant aggregates on uptake

The results of the experiments suggest a possible role/effect of the large aggregates present in exogenous surfactant. To determine whether the uptake was stimulated by the large or small aggregates of surfactant, experiments were performed as shown in Figure 1.

Addition of exogenous surfactant in a fluorescent liposome/surfactant ratio of 1:50 (Group 1) resulted in a significant increase in uptake of fluorescent labeled liposomes by alveolar type II cells (Fig. 3A) and alveolar macrophages (Fig. 3C), as well as an increase in the number of alveolar type II cells involved in the uptake (Fig. 3B). In Group 2, treated with 'large aggregates', an increase in the uptake of fluorescent labeled liposomes by alveolar type II cells (Fig. 3A) was observed, as well as an increase in the number of alveolar type II cells involved in the uptake, in comparison to the incubation without any surfactant preparation (Normal) (Fig. 3B). Also, the addition of these 'large' aggregates to alveolar macrophages resulted in a similar significant increase in the uptake of fluorescent liposomes (Fig. 3C) although the number of macrophages taking up liposomes was unaffected (Fig. 3D). In Group 3, containing 'small' aggregates, no effects on the uptake of fluorescent labeled liposomes or on the cell number involved in the uptake was observed for alveolar cells (Fig. 3).

In order to determine whether the fluorescent liposomes associate with the surfactant large aggregates and thereby facilitating uptake of this whole complex or large aggregates stimulate the uptake of small aggregates by alveolar cells, exogenous surfactant was suspended in a suspension containing fluorescent labeled liposomes (Group 4 and 5) which was followed by centrifugation. Of the resulting pellet and supernatant a total amount of 50 µl was added to the incubation. The addition of the pellet (Group 4) resulted in a significantly lower cell-associated fluorescence of both alveolar type II cells and alveolar macrophages (Fig. 3A, C). In addition, the percentage of cells that were involved in the uptake of fluorescent liposomes was also significantly lower in comparison to normal liposomes.

On the other hand, the addition of the supernatant (Group 5), did not affect the uptake of labeled liposomes by either alveolar type II cells or alveolar macrophages, as all values, mean cell-associated fluorescence and number of cells taking up liposomes did not differ significantly from those derived by the addition of only fluorescent labeled liposomes (Normal).

Effect of exogenous surfactant on the uptake *in vivo*

To study if the effects of exogenous surfactant on the uptake of surfactant-like liposomes were also present *in vivo*, healthy, ventilated rats were intratracheally instilled with fluorescent labeled liposomes and exogenous surfactant.

Exogenous surfactant was suspended in a suspension containing fluorescent labeled liposomes (1 mg/ml) to a liposome to surfactant ratio of 1:50.

Instillation of this suspension in ventilated animals did not affect the uptake of labeled liposomes, compared to the uptake of the same labeled liposomes without exogenous surfactant (Fig 4A). However, the percentage alveolar type II cells involved in the uptake was significantly decreased compared to the 'normal' liposomes, that is without exogenous surfactant (Fig 4B). On the other hand, the uptake of this suspension containing both exogenous surfactant and

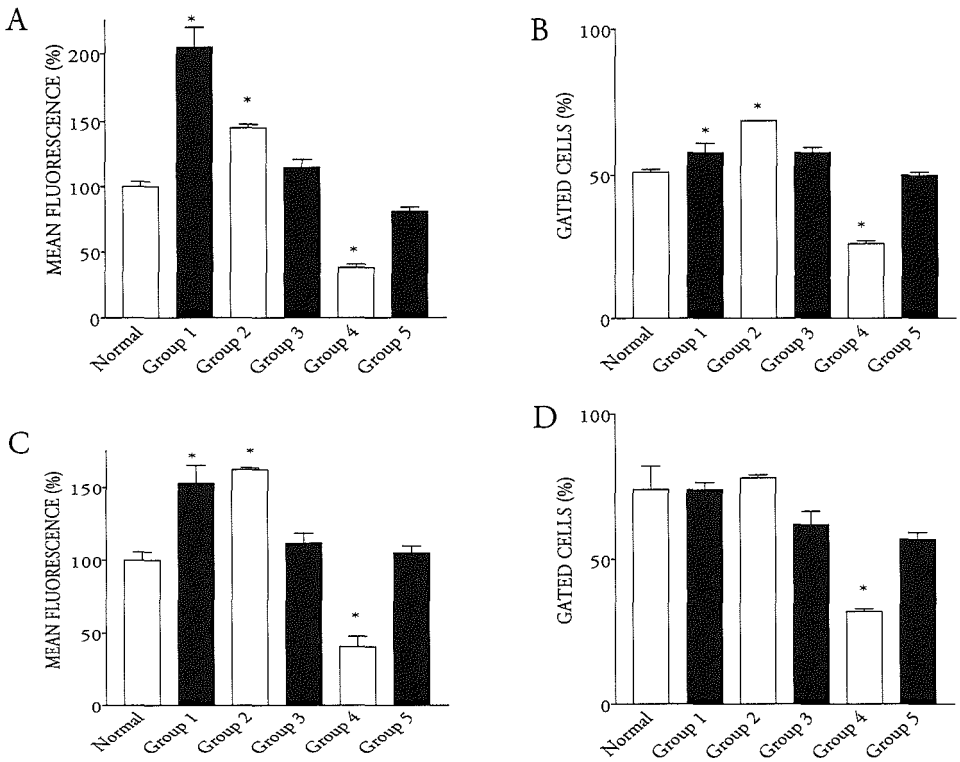


Figure 3. *Effect of large and small surfactant aggregates on uptake in vitro*
Alveolar cells were isolated from untreated animals and a total of $3*10^5$ alveolar type II cells (A, B) or alveolar macrophages (C, D) were incubated at 37°C for one hour. The 'Normal' group received only 25 µg/ml fluorescent labeled liposomes. Exogenous surfactant was added in a liposome to surfactant ratio of 1:50 (Group 1) together with 25 µg/ml fluorescent labeled liposomes. Centrifuged surfactant (27,000*g) was added in a similar liposome to surfactant ratio of 1:50 (pellet of the surfactant in Group 2, supernatant in Group 3). A mixture of fluorescent labeled liposomes and exogenous surfactant in (again) a liposome to surfactant ratio of 1:50 was centrifuged and both pellet (Group 4) and supernatant (Group 5) were incubated with alveolar cells. Flow cytometry was used to determine cell-associated fluorescence (A, C) and percentage of cells involved in the uptake of the labeled liposomes (B, D) (n = 5 incubations per group; values are expressed as mean ± SEM). * indicates a significant difference compared with the Normal group.

labeled liposomes did not influence the uptake by alveolar macrophages or the percentage alveolar macrophages involved in the uptake when compared to the normal liposomes (Fig. 4C, D).
More interestingly, however, when the same experiments were repeated with sonicated, inactivated, exogenous surfactant, similar results were found. The administration of a suspension containing sonicated surfactant and labeled liposomes did not change the uptake of labeled liposomes by alveolar type II cells or alveolar macrophages in comparison to the

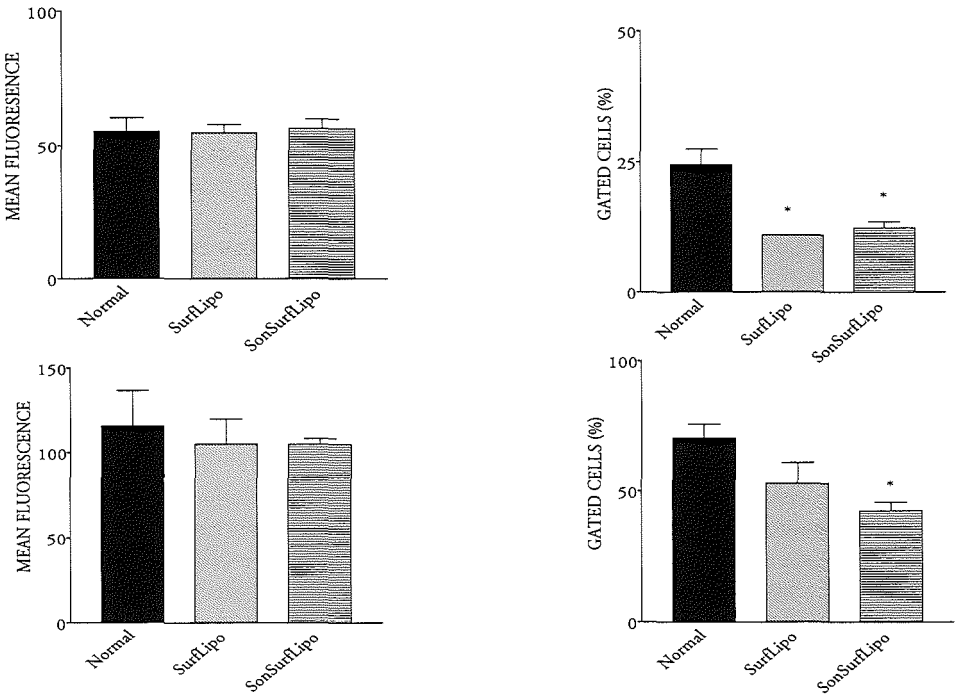


Figure 4. *Effect of exogenous natural surfactant on uptake in vivo*
The animals were randomly divided into 3 groups. All animals were intratracheally instilled with fluorescent labeled liposomes (3 mg/kg). Depending on the group, either exogenous natural surfactant (Surf)(150 mg/kg) or sonicated exogenous surfactant (SonSurf)(150 mg/kg) was intratracheally administered together with the liposomes (SurfLipo, SonSurfLipo). One additional group received no surfactant (Normal). One hour after instillation of the fluorescent labeled liposomes, the animals were sacrificed and alveolar cells were isolated. Cell associated fluorescence (A, C) and percentage of cells involved in the uptake of liposomes (B, D) was determined using flow cytometry (n = 5 animals per group; values expressed as mean ± SEM). * indicates a significant difference compared to the normal liposomes.

uptake of only labeled liposomes. Nevertheless, both the percentage of alveolar type II cells and alveolar macrophages involved in the uptake of labeled liposomes was significantly decreased by the addition of sonicated surfactant, compared to the percentage of alveolar cells taking part in the uptake when using 'normal' liposomes (Fig. 4B, D).

Localization of cell-associated fluorescence
To ascertain that the cell-associated fluorescence was located within the cell rather than binding to the outer membrane of the cell, confocal laser microscopy was used. The confocal scans through the middle of the cell show a punctuate fluorescence throughout the cell limited to its circumference with the exception of its nucleus, demonstrating the intracellular presence of the fluorescent liposomes for both alveolar type II cell (Fig 5A) and alveolar macrophage (Fig. 5B).

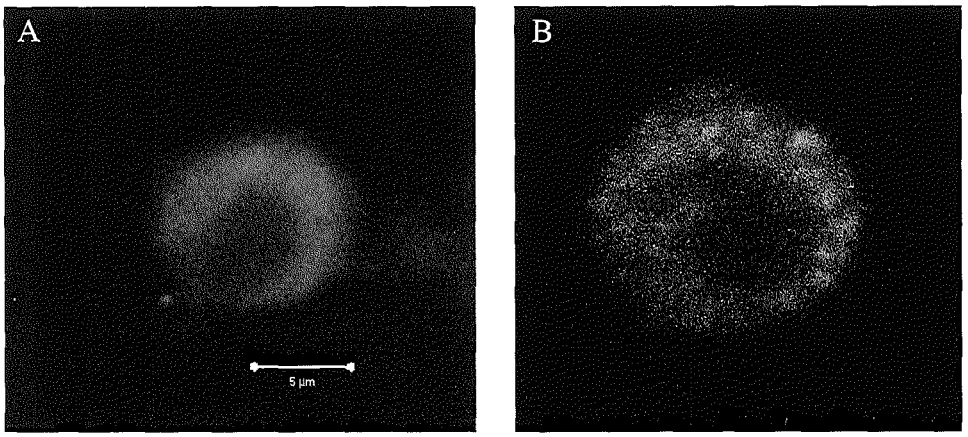


Figure 5. *Localization of cell-associated fluorescence*
Both alveolar type II cells (A) and alveolar macrophages (B) were isolated from untreated animals and incubated with 25 µg/ml fluorescent labeled liposomes and exogenous surfactant (liposome/surfactant ratio 1:50). One hour after incubation, cells were washed 3 times with cold PBS and the cells were mounted on glass coverslips.

Discussion

Pulmonary surfactant has been widely accepted to be essential for normal breathing, that is with physiological pressures. Surfactant facilitates this by providing a dynamic, low surface tension at the alveolocapillary membrane in the lung. Unfortunately, this endogenous surfactant can be absent or decreased, as in premature infants, or inactivated by a variety of causes, leading to respiratory failure. The use of exogenous surfactant is nowadays widely accepted in clinical practice, mostly in neonates, though its use in adults is under investigation[5], and improves outcome significantly.
The administration of large amounts of exogenous surfactant might influence the surfactant metabolism, possibly via a feedback mechanism. The endogenous surfactant metabolism consists of production, secretion, formation of lipid monolayer, turnover, re-uptake and recycling. Several studies have focused on the effects of exogenous surfactant on the production and secretion[16-19]. However, little is known about the influence of large amounts of exogenous surfactant on the uptake, as the few studies performed focused on the clearance of the surfactant or individual components and their results are contradictory[18, 20-25]. This re-uptake of surfactant lipids has been demonstrated to be essential, especially in neonates, who have been demonstrated to rely on recycling for >90%, indicating the need for uptake of inactivated surfactant[11].
Pulmonary surfactant consists of large and small aggregates; it is thought that the large aggregates are surface active and lower surface tension. These 'large' aggregates are then turned over into 'small' aggregates, which are incapable of providing a variable surface tension and are cleared from the alveolar space by alveolar type II cells and alveolar macrophages and, if possible, recycled.
In the current study we investigated the effects of exogenous surfactant on the uptake of liposomes with a composition similar to small aggregates, both *in vitro* and *in vivo*. In addition, we studied whether any effects caused by exogenous surfactant were induced by large or small aggregates.

From the *in vitro* experiments it can be concluded that exogenous surfactant influences the uptake of labeled liposomes. The addition of exogenous surfactant in a liposome to surfactant ratio of 1:50 increases the uptake in both alveolar type II cells and alveolar macrophages. More specifically, the 'large' aggregates enhance the uptake of fluorescent labeled surfactant-like liposomes. However, it has been demonstrated that DPPC liposomes associate with large aggregates[26], though in the current experiments it is unlikely that the large aggregates associated with the fluorescent labeled liposomes. We demonstrated that addition of a suspension (created by centrifuging a solution containing exogenous surfactant and fluorescent liposomes and resuspending the pellet), to alveolar cells induced a significantly lower cell-associated fluorescence of these alveolar cells compared to alveolar cells incubated with only fluorescent labeled liposomes.

This stimulation of the uptake of labeled liposomes by alveolar cells by exogenous surfactant was also investigated *in vivo*. Interestingly, the cellular uptake of labeled liposomes was unaffected by the administration of exogenous surfactant. However, the percentage alveolar type II cells was significantly decreased compared to normal liposomes. Thus, it might be suggested that *in vivo*, exogenous surfactant downregulates the uptake by decreasing the percentage of cells involved in the uptake and most likely recycling; a negative feedback mechanism. It has been demonstrated that in injured lungs surfactant synthesis is increased[27, 28], and in addition uptake is increased in injured lungs[29]. In injured lungs, surfactant function is decreased, which has to be compensated for. Additional surfactant in healthy animals may lead to a downregulation of the surfactant metabolism, with less need for recycling; *de novo* synthesis is sufficient as only little new surfactant is needed. *In vitro*, however, *de novo* synthesis is unlikely because no substrate is available and the cells rely on recycling and thus uptake. Surfactant stimulates the cell to produce surfactant, ensuring a constant amount of surfactant in the 'alveolar' space. This stimulation is induced by the large aggregates, as demonstrated in the current study.

The instillation of sonicated surfactant induces an effect similar to surface active surfactant. However, the *in vitro* experiments suggested competition between sonicated surfactant and labeled liposomes. Therefore, an explanation for the *in vivo* results is more complex. It is unlikely that only competition between the sonicated surfactant and labeled liposomes takes place as only the number of cells involved in the uptake is decreased, whereas the uptake per cell remains at the same level. This unexpected behavior of sonicated surfactant underlines the hypothesis of feedback mechanisms via hormonal, inflammatory or nerval parameters, present *in vivo* though absent *in vitro*. However, further studies are needed to clarify the feedback mechanism(s) *in vivo* and the effects of exogenous surfactant in diseased lungs.

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CHAPTER 7

Effect of SP-B analogues on the uptake of liposomes by alveolar cells *in vivo*

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Submitted

Abstract

Background: The in vivo effect on uptake of liposomes by synthetic SP-B analogues was investigated by measuring their effect on the uptake of surfactant-like liposomes by alveolar cells. Native SP-B and four SP-B analogues were included: monomeric and dimeric SP-B_{1-25} (Cys-11>Ala-11), both these peptides have surface tension lowering activities though the dimeric form is more active, and monomeric and dimeric Serine SP-B_{1-25} (Cys-11>Ala-11 and Arg-12>Ser-12) which do not have surface tension lowering activities.
Method: Healthy, mechanically ventilated rats received intratracheally various SP-B peptides (SP-B/lipid ratio 1:33 w/w) incorporated in surfactant-like liposomes (consisting of DPPC, PC, PG, PI, cholesterol and fluorescent labeled PE in a weight ratio of 55:21:8:2:8:6). One hour after instillation, alveolar macrophages (AM) and alveolar type II (ATII) cells were isolated and cell-associated fluorescence was determined using flow cytometry. Confocal laser microscopy was performed to ensure internalization of the liposomes rather than association of the liposome with the outer cell membrane.
Results: The amount of cell-associated fluorescence of AM was the same for all liposome preparations, including the four with SP-B peptides as well as the control liposome preparation which did not contain SP-B. However, cell-associated fluorescence of ATII cells in the presence of liposomes incorporated with the serine SP-B peptides was reduced by 40% compared to the control liposomes. Confocal microscopy demonstrated the internalization of all liposome preparations by AM and ATII cells.
Conclusion: Uptake of liposomes by ATII cells of liposomes with monomeric and dimeric SP-B_{1-25} was similar to the uptake of liposomes with native SP-B, but decreased by the addition of serine SP-B. The incorporation of SP-B analogues results in an increased uptake of these liposomes by alveolar macrophages for monomeric SP-B_{1-25} and serine dimeric SP-B_{1-25} whereas dSP-B_{1-25} results in a decreased uptake.
Therefore, mutant SP-B peptides, such as serine SP-B, may depress surfactant recycling and thus affect surfactant metabolism.

Introduction

Pulmonary surfactant is essential for normal breathing, and its absence leads to respiratory failure. The presence of endogenous surfactant in the lung is dependent upon a very complex system of production, secretion, formation of lipid monolayer, turnover, re-uptake and recycling. Briefly, after secretion, surfactant unfolds to tubular myelin that is incorporated into the lipid monolayer. During respiration the surfactant in the lipid monolayer is turned over from large surface active aggregates into small inactive surfactant aggregates[1-3]. These small aggregates are removed from the alveolar space by both alveolar macrophages and alveolar type II cells. As *de novo* synthesis is insufficient to compensate the loss of surfactant, especially in neonates, these small aggregates are recycled by the alveolar type II cells.
The uptake of small aggregates is influenced by several factors, such as environmental ones[4], lipid constitution and surfactant proteins, mainly SP-A, SP-B and SP-C. Especially SP-A and to a smaller extent SP-C have been demonstrated to stimulate the uptake of surfactant lipids by alveolar type II cells and macrophages[5-13]. The effects of SP-B on the uptake are, however, more complex; it ahs been shown that SP-B can increase the uptake of liposomes *in vitro*[10, 14-17], whereas others have demonstrated an inhibition of SP-B on SP-C mediated uptake by alveolar type II cells[9, 10].
Exogenous surfactant has been accepted worldwide as a therapy of RDS in premature and term infants, and the use of surfactant therapy for adults is under discussion. Exogenous surfactant

is usually derived from lung extracts containing phospholipids and the two hydrophobic surfactant proteins SP-B and SP-C; both these surfactant proteins play an important role in the surface tension lowering properties of surfactant[18-20]. Moreover, the need of the two proteins is emphasized by the fact that absence of SP-B is lethal[21, 22], whereas SP-C is not but mutations in the SP-C gene can be related to interstitial lung diseases[23].
Synthetic analogues of SP-B and SP-C based on the known human amino-acid sequence are nowadays tested with the aim to develop a completely synthetic surfactant preparation. Nevertheless, the effects of the surfactant proteins on the endogenous surfactant metabolism remain unknown.
Our laboratory has been able to formulate and synthesize synthetic SP-B and SP-C peptides, which closely mimic the function of natural surfactant proteins[24]. The *in vivo* metabolism and the effects on the clearance of these surfactant peptides have yet not been investigated. We studied the effect of monomeric and dimeric SP-B_{1-25} peptides, based on the N-terminal segment of human SP-B with an alanine for cysteine substitution at position 11, on the uptake of surfactant-like liposomes by alveolar type II cells and alveolar macrophages. The SP- B_{1-25} (Cys-11 > Ala-11) variant disulfide linked homodimer has been the focus of recent laboratory studies as it closely mimics the *in vitro* and *in vivo* functions of the full-length SP-B protein[25-27].

Materials and methods

Ethical Guidelines
This study was approved by the Institutional Animal Care and Use Committee at the Erasmus MC - Faculty Rotterdam.

Porcine SP-B and synthetic SP-B analogues
Porcine SP-B was isolated from lung lavage and characterized according to standard procedures[28]. Monomeric SP-B_{1-25} peptide was synthesized on a 0.25 mmol scale with an Applied Biosystems Model 431A peptide synthesizer using a FastMoc™ strategy[29]. The SP-B_{1-25} sequence was based on the N-terminal of human SP-B[30] with one modification, cysteine in position 11 was replaced by alanine (Cys-11 > Ala-11 variant monomer)[25, 26]. The peptide was synthesized with prederivatized Fmoc-Gly resin (CalbiochemNova, La Jolla) or PEG-PA resin (Perceptive Biosystems, Old Connecticut Path, MA) and single coupled for all residues. After purification by reversephase high performance liquid chromatography, the molecular mass was confirmed by fast atom bombardment mass spectroscopy or electro spray mass spectroscopy[30]. The SP-B_{1-25} (Cys-11 > Ala-11) variant disulfide linked homodimer was formed by oxidizing the monomeric SP-B_{1-25} peptide (mSP-B_{1-25})[25, 26]. The molecular mass of this dimeric SP-B_{1-25} peptide (dSP-B_{1-25})was confirmed by electro spray mass spectroscopy and indicated the yield of dimeric product to be essentially 100%[25].
Mutant monomeric and dimeric SP-B_{1-25} peptides were synthesized with site-specific substitutions of serine for arginine in positions 12 and 17 and for lysine in positions 16 and 24 of the N-terminal (Fig. 1). These site-specific substitutions reduce the net cationic charge on the molecule without affecting the hydrophobicity[24]. Synthesis, purification and characterization of the serine mutants were done in the same manner as the original peptides.
All peptides were produced in their native state and then their N-terminal was fluorescent labeled on the resin with Bodipy® FL, CASE (N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)cysteic acid, succinimidyl ester, triethylammonium salt) (Molecular Probes Inc., Eugene, OR, USA) using a previously reported protocol[31]. The N-terminal labeled peptides were cleaved and deprotected from the resin by Hydrogen Fluoride

SP-B1-25 monomer
Phe-Pro-Ile-Pro-Leu-Pro-Tyr-**Cys**-Trp-Leu-Ala-Arg-Ala-Leu-Ile-Lys-Arg-Ile-Gln-Ala-Met-Ile-Pro-Lys-Gly

$SP\text{-}B_{1\text{-}25}$ *serine monomer*
Phe-Pro-Ile-Pro-Leu-Pro-Tyr-**Cys**-Trp-Leu-Ala-Ser-Ala-Leu-Ile-Ser-Ser-Ile-Gln-Ala-Met-Ile-Pro-Ser-Gly

Figure 1. *Peptide sequences of the SP-B analogues and their serine mutants*
The $SP\text{-}B_{1\text{-}25}$ homodimer consists of two $SP\text{-}B_{1\text{-}25}$ monomers which are disulfide-linked at Cys8. The fluorescent label was inserted in all peptides at the N-terminus, shown on the left side of the sequence.

methodology[32]. The crude labeled peptides were then purified by reverse phase HPLC[26] and their mass confirmed by MALDI-TOF mass spectrometry (UCLA mass spectometry facility).

Liposome preparation
Surfactant-like liposomes were prepared by mixing the following lipids; dipalmitoyl phosphatidylcholine (DPPC), phosphatidyl choline (PC), phosphatidylglycerol (PG), phosphatidyl inositol (PI), and phosphatidyl ethanolamine (PE) labeled with Texas-Red in the head group (Texas-Red DHPE, Molecular Probes Inc., Leiden, The Netherlands) and cholesterol in a weight ratio of 55:21:8:2:6:8 and the indicated surfactant proteins at a protein/lipid concentration of 3:100 wt:wt. Subsequently, this mixture was dried under a stream of nitrogen. The lipids were purchased from Sigma (Zwijndrecht, The Netherlands), unless stated otherwise. The liposomes were suspended in PBS at a concentration of 0.5 mg lipids/ml (*in vitro* experiments) or 1 mg/ml (*in vivo* experiments) using glass pearls and vortexing. Immediately prior to use, the liposome suspension was sonicated for 2 min on ice using an ultrasonic disintegrator (Branson Sonifier 250, Danbury, CT, USA) to prepare small unilamellar liposomes[4]. The size of the liposomes was determined by dynamic light scattering at 25°C with a Malvern 4700 system using a 25 mW He-Ne laser (NEC, Tokyo, Japan) and the auto measure version 3.2 software (Malvern Ltd, UK). As a measure of particle size distribution of the dispersion, the system reports a polydispersity index (p.d.). This index ranges from 0.0 for a monodisperse and up to 1.0 for an entirely polydisperse dispersion. After ultrasonification the liposomes size ranged from 140 to 165 nm and the polydispersity index ranged from 0.2 to 0.35.

Intratracheal instillation of fluorescent liposomes
The studies were performed with male Sprague-Dawley rats (IFFA Credo, The Netherlands) with a bodyweight of 318 ± 16 g. After induction of anesthesia with a mixture of nitrous oxide (66%), oxygen (33%) and isoflurane (1-2%), a sterile polyethylene catheter (0.8 mm o.d.) was inserted into one of the carotid arteries. The animals were then tracheotomized and a sterile metal cannula was inserted into the trachea.
After these surgical procedures, gaseous anesthesia was ended and replaced with an intraperitoneal injection of pentobarbital sodium (60 mg/ml, Nembutal®, Algin BV, Maassluis, The Netherlands) at a dose of 30 mg/kg bodyweight every hour.
Muscle relaxation was induced and maintained by an hourly intramuscular injection of pancuronium bromide (2 mg/kg, Pavulon; Organon Technika, Boxtel, The Netherlands). The

animals were then mechanically ventilated with a Servo® ventilator 900C (Siemens-Elema, Solna, Sweden) set to pressure control mode using a frequency of 30/min, an inspiratory/ expiratory ratio of 1:2, a positive end-expiratory pressure (PEEP) of 2 cm H_2O, a peak inspiratory pressure (PIP) of 12 cm H_2O and FiO_2 was set to 1.
Before instillation of the labeled liposomes, PEEP was increased to 6 cm H_2O and PIP was increased to 26 cm H_2O. After disconnection from the ventilator, the liposomes were administered intratracheally at the indicated dosage. The suspension of liposomes (1 mg lipids/ml) was administered as a bolus of 3 ml/kg followed by a bolus of air (12 ml/kg), directly into the endothracheal tube via a syringe, and the animals were immediately reconnected to the ventilator. Thirty minutes after instillation of the liposomes PEEP was reduced to 2 cm H_2O and PIP to 12 cm H_2O.
Arterial blood gas values were measured with conventional methods (ABL 555, Radiometer, Copenhagen, Denmark) at the start of ventilation, immediately after instillation of the liposomes and every 30 minutes thereafter. After one hour of ventilation the animals were sacrificed by exsanguination via the abdominal aorta and the alveolar type II cells and alveolar macrophages were isolated to determine the cell-associated fluorescence. Untreated animals were sacrificed immediately after anesthesia and their isolated alveolar type II cells and alveolar macrophages were used to correct for auto-fluorescence.

Isolation of alveolar type II cells and alveolar macrophages
Prior to isolation of the cells, the thorax was opened and blood was removed from the lungs by perfusing the pulmonary artery with warmed saline (37°C) supplemented with 20 IE heparin per ml (Leo Pharma, Weesp, The Netherlands). The lungs were removed en bloc from the thoracic cavity and lavaged with 10 ml of solution 1 [140 mM NaCl, 5 mM KCl, 2.5 mM $Na_2HPO_4.2H_2O$, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethaesulfonic acid), 6 mM glucose, 0.2 mM EGTA (ethylene glycol-bis- (β-amino ethyl ether) N,N'-tetra-acetic acid), pH 7.40] at 37°C. This procedure was repeated four times. The lung lavages were pooled per animal and centrifuged (100x g; 10 min; 4°C). The cellular pellet, i.e. alveolar macrophages, was suspended in solution 2 [140 mM NaCl, 5 mM KCl, 2.5 mM $Na_2HPO_4.2H_2O$, 10 mM HEPES, 6 mM glucose, 2.0 mM $CaCl_2$ and 1.3 mM $MgSO_4$] to a concentration of $2x10^6$ cells/ml and stored on ice until further use. The alveolar type II cells were isolated according to Dobbs et al.[33], using enzymatic digestion as previously described. Alveolar type II cells were suspended in solution 2 at a concentration of $2x10^6$ cells/ml and stored on ice until further use. Alveolar macrophages were identified using monoclonal antibodies specific for rat macrophages (ED9) and alveolar type II cells were identified using an alkaline phosphatase assay as described by Edelson et al.[34]. The average yield of alveolar type II cells was $16x10^6$ with a purity of 80 ± 5% and $5x10^6$ alveolar macrophages with a purity of 92 ± 5% per rat.

In vitro uptake of SP-B peptide
Alveolar cells of untreated animals were isolated as described above and suspended in solution 2 to a concentration of $2x10^6$ cells/ml. A total of $3x10^5$ cells were incubated with the indicated concentration of the specified liposomes at 37°C (final volume 500 µl) in a shaking water bath. After 1 hour, the incubation was terminated by addition of 2 ml of ice-cold PBS (4°C). The cell-suspension was centrifuged at 100 x g for 10 min at 4°C. The supernatant was removed, and the cells were suspended in 2 ml ice-cold PBS and centrifuged again. This wash procedure was repeated twice. Finally, the pellet was resuspended in 200 µl of cold PBS, and cell-associated fluorescence was determined as described below.

Flow cytometry
Cell-associated fluorescence of the alveolar type II cells and alveolar macrophages as a measure

for internalized liposomes was determined using flow cytometry (FACSCalibur; Becton Dickinson, Mountain View, CA, USA). The cell-associated fluorescence of 15,000 cells was determined. Alveolar macrophages and alveolar type II cells derived from untreated control animals were used in each experiment to determine the auto-fluorescence of the cells. Subsequently, the mean cell-associated fluorescence was determined only for those cells that had a higher fluorescence than that caused by auto-fluorescence (gated cells).

Localization of cell-associated fluorescence
To localize the cell-associated fluorescence, intracellular or extracellular, confocal laser micrographs were obtained using a confocal microscope (LSM510NLO, Carl Zeiss, Jena, Germany). Images were created with plan apochromat 63x 1.4 n.a., objectives and photomultiplier tubes and filters set at 500-550 nm and 656-615 nm (TexasRed, excitation 596 nm; Bodipy, excitation 503 nm HeNe laser). Images of alveolar cells were serially sectioned pinhole at 1 airy unit with an interval of 0.5 μm to distinguish cell membrane-associated fluorescence from the intracellular fluorescence.

Statistical analysis
Differences between the 6 groups that received liposomes with a different composition were analyzed using an ANOVA followed by a Bonferroni post-hoc test. Blood gas values were analyzed using a repeated measurement ANOVA. Differences were considered statistically significant at a $p < 0.05$. Values are expressed as mean ± SEM.

Results

Effect of SP-B_{1-25} on the uptake of liposomes by alveolar cells in vitro
After isolation from untreated animals, alveolar cells were incubated with different concentrations of liposomes. In these liposomes, native SP-B (nSP-B), monomeric SP-B_{1-25} or dimeric SP-B_{1-25} was included in a protein to lipid ratio of 3:100. After 1 hour the incubation was stopped and cell-associated fluorescence was determined.
The incorporation of SP-B (or derivations) in the fluorescent labeled liposomes did not influence the uptake of these liposomes by alveolar cells (Fig. 2 A, C). Nor was the percentage of cells involved in the uptake affected by the inclusion of proteins in the liposome (Fig 2 B, D).

Effect of SP-B_{1-25} analogues containing liposomes on the gas exchange
The inclusion of SP-B_{1-25} analogues in the labeled liposomes did not affect gas-exchange. Arterial blood gas values remained at healthy levels after instillation (results not shown).

Effect of SP-B_{1-25} peptides on the uptake by alveolar cells in vivo
To study the effect of SP-B analogues, the different SP-B peptides were incorporated in fluorescent labeled liposomes at a protein to lipid concentration of 3:100 wt:wt. The uptake by alveolar type II cells of labeled liposomes was significantly increased with the incorporation of dSP-B_{1-25} whereas the inclusion of other SP-B analogues did not affect uptake (Fig. 3A). However, the percentage of alveolar type II cells involved in the uptake decreased when dSP-B_{1-25} was incorporated, while the inclusion of mSP-B_{1-25} increased the percentage of alveolar type II cells (Fig. 3B). Such an effect of the inclusion on the percentage of cells involved in the uptake of labeled liposomes was also observed for alveolar macrophages. The percentage alveolar macrophages taking part in the uptake was significantly decreased with the inclusion of dSP-B_{1-25} in the liposome. In addition, the incorporation of serine mSP-B_{1-25} also

decreased the amount of alveolar macrophages taking up labeled liposomes (Fig. 3D). Interestingly, the uptake per alveolar macrophage was not affected by the incorporation of dSP-B_{1-25} or serine mSP-B_{1-25}, but was significantly increased due to the incorporation of mSP-B_{1-25} or serine dSP-B_{1-25} (Fig. 3C). These results raise the question as to how total uptake was affected by inclusion of the SP-B analogues, and therefore total cell-associated fluorescence was determined, which takes into consideration both the uptake per cell as well as the percentage of cells involved in the uptake. These data demonstrate a decreased uptake of liposomes with serine SP-B_{1-25} analogues by alveolar type II cells (Fig. 4A). The incorporation of serine mSP-B_{1-25} did not influence uptake by alveolar macrophages. However, inclusion of serine dSP-B_{1-25} resulted in a significant increase in uptake of labeled liposomes by alveolar macrophages similar to the increase caused by mSP-B_{1-25} (Fig 4B), whereas enclosure of dSP-B_{1-25} in the labeled liposomes resulted in a decreased uptake by alveolar macrophages.

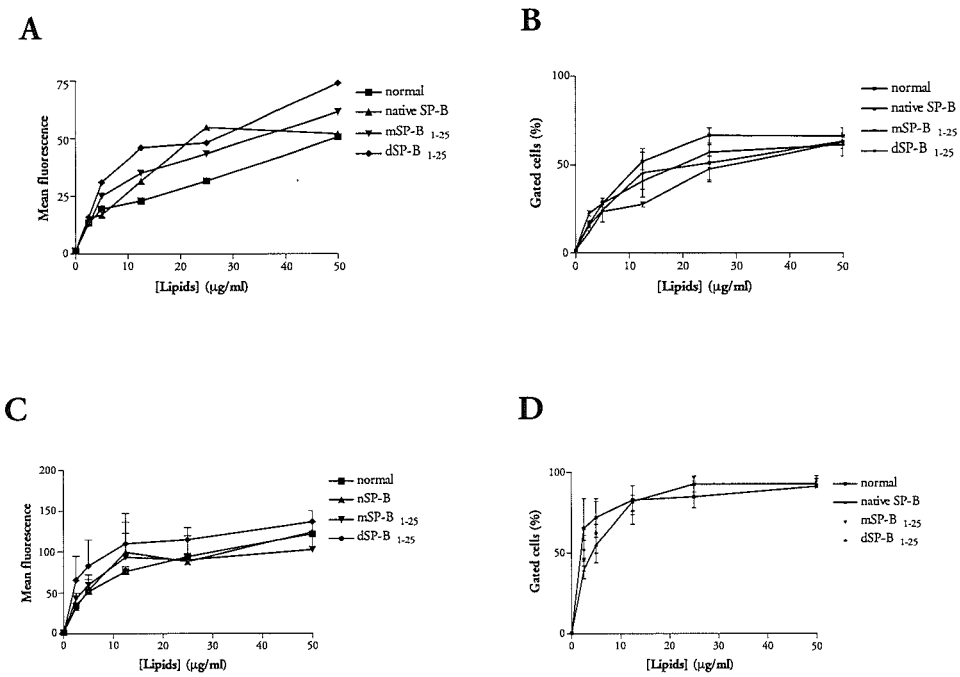

Figure 2. *Effect of nSP-B, mSP-B_{1-25} and dSP-B_{1-25} on the alveolar cell uptake in vitro*
Alveolar cells were isolated from untreated animals and incubated for 1 hour at 37°C with the indicated concentrations of fluorescent liposomes dependent on the group containing native SP-B (nSP-B), monomeric SP-B_{1-25} (mSP-B_{1-25}) or dimeric SP-B_{1-25} (dSP-B_{1-25}) or no proteins (normal). The SP-B proteins were included in a protein to lipid ratio of 3:100. The mean cell-associated fluorescence of gated alveolar type II cells (A) and alveolar macrophages (C) was determined. In addition, the percentage gated alveolar type II cells (B) and alveolar macrophages (D) were determined. No significant differences were observed at any concentration (n = 4 incubations at every concentration; values are mean ± SEM).

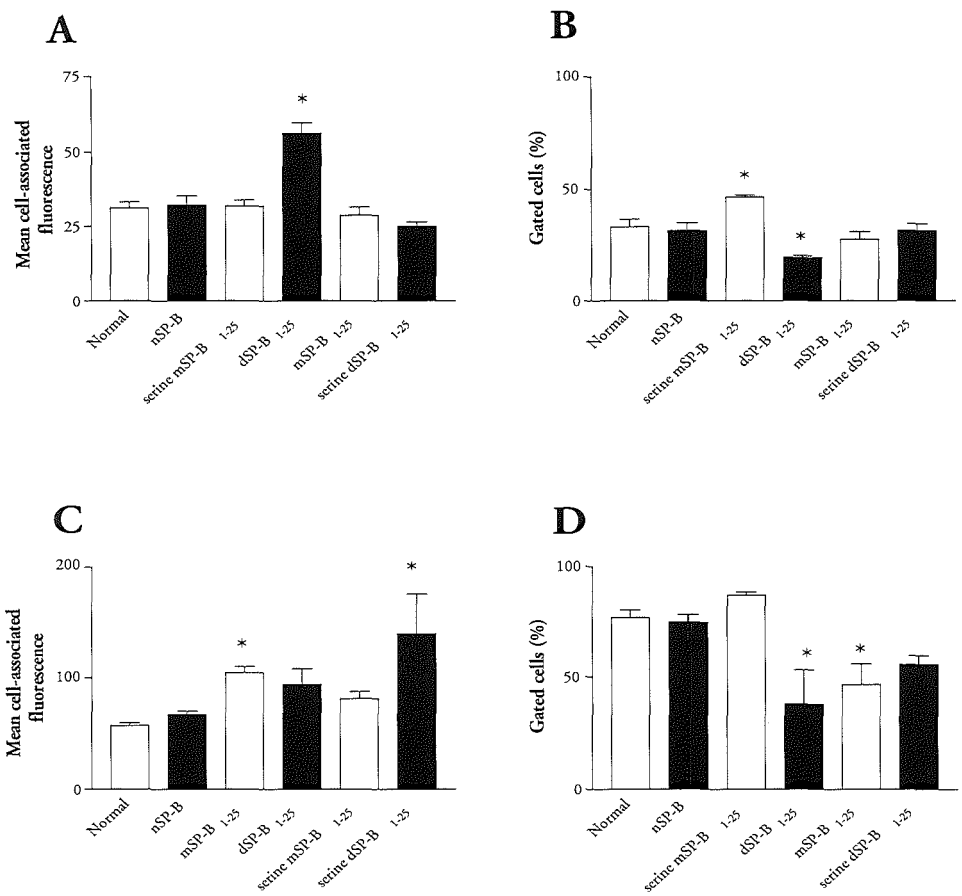

Figure 3. *Effect of SP-B and SP-B analogues on the alveolar cell uptake in vivo*
Fluorescent labeled liposomes with native SP-B (nSP-B), monomeric SP-B_{1-25} (mSP-B_{1-25}), dimeric SP-B_{1-25} (dSP-B_{1-25}), serine monomeric SP-B_{1-25} (serine mSP-B_{1-25}) or serine dimeric SP-B_{1-25} (serine dSP-B_{1-25}) incorporated at a protein to lipid ratio of 3:100 were intratracheally instilled in ventilated rats. After one hour, alveolar type II cells (A, B) and alveolar macrophages were isolated (C, D), and cell-associated fluorescence (A, C) and percentage cells involved in the uptake (B, D) were determined. (n = 4 rats per group; values are mean ± SEM); *, $p<0.05$ versus normal

Localization of cell-associated fluorescence caused by SP-B_{1-25}
To ascertain that the cell-associated fluorescence was located within the cell rather than binding to the outer membrane of the cell, confocal laser scanning microscopy was used. One hour after intratracheal instillation of the liposomes containing mSP-B_{1-25} or dSP-B_{1-25} in mechanically ventilated rats, the alveolar cells were isolated and confocal scans were made. These confocal scans through the middle of the cell show a punctuate fluorescence throughout the cell limited to its circumference with the exception of its nucleus, demonstrating the intracellular presence of the fluorescent liposomes (Fig 5 and 6).

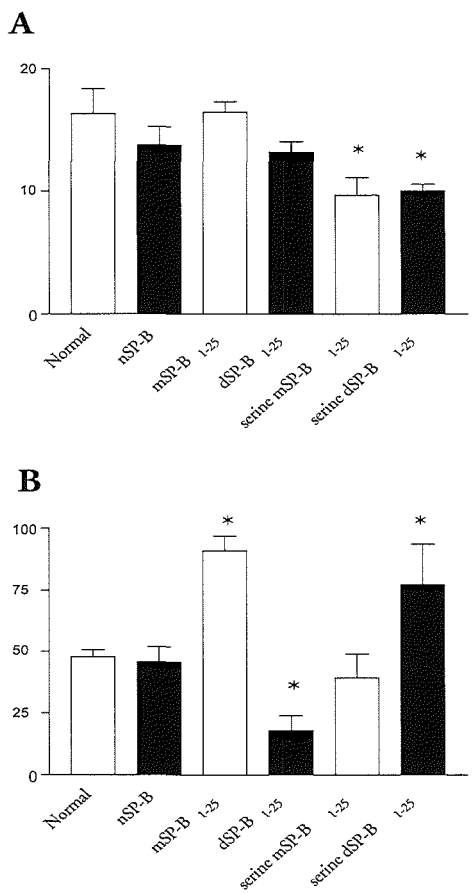

Figure 4. *Effect of SP-B_{1-25} on total uptake of liposomes*
Fluorescent labeled liposomes with native SP-B (nSP-B), monomeric SP-B_{1-25} (mSP-B_{1-25}), dimeric SP-B_{1-25} (dSP-B_{1-25}), serine monomeric SP-B_{1-25} (serine mSP-B_{1-25}) or serine dimeric SP-B_{1-25} (serine dSP-B_{1-25}) incorporated at a protein to lipid ratio of 3:100 were intratracheally instilled in ventilated rats. After one hour, alveolar type II cells (A) and alveolar macrophages (B) were isolated, and total cell-associated fluorescence was determined. (n = 4 rats per group; values are mean ± SEM); *, $p<0.05$ versus normal

Discussion

SP-B is essential for the biophysical properties of pulmonary surfactant. SP-B is a 79 amino acid, cysteine rich hydrophobic protein found in pulmonary surfactant as an 18 kDa dimer. It is synthesized in the endoplasmic reticulum of the alveolar type II cell as a large precursor protein of 42 kDa. This precursor SP-B is transported via the Golgi complex and the multivesicular bodies to the lamellar bodies while it undergoes proteolytic processing to the mature 9 kDa protein. In the lamellar bodies SP-B combines with the other surfactant proteins and lipids. The lamellar bodies are secreted into the alveolar space, where they provide functional surfactant for the air-water interface. The surfactant metabolism *in vivo* has recently been reviewed[35]. In the adult lung SP-B is cleared more rapidly from the alveoli than saturated phosphatidylcholine[36]. In the developing lung, the clearance of phospholipids and SP-A is in general much slower than in the adult lung[37, 38]. The clearance of SP-A, B, and C has been studied in adult (rabbit) lungs[36, 37, 39], the clearance of SP-B in newborn rabbits[40] and that of SP-C in preterm lambs[41]. The surfactant proteins are not only cleared from the alveolar space but may also play a role in the clearance of surfactant lipids from the alveolar space. Both SP-A and SP-C have been shown to stimulate the uptake. The effects of SP-B are more complex,

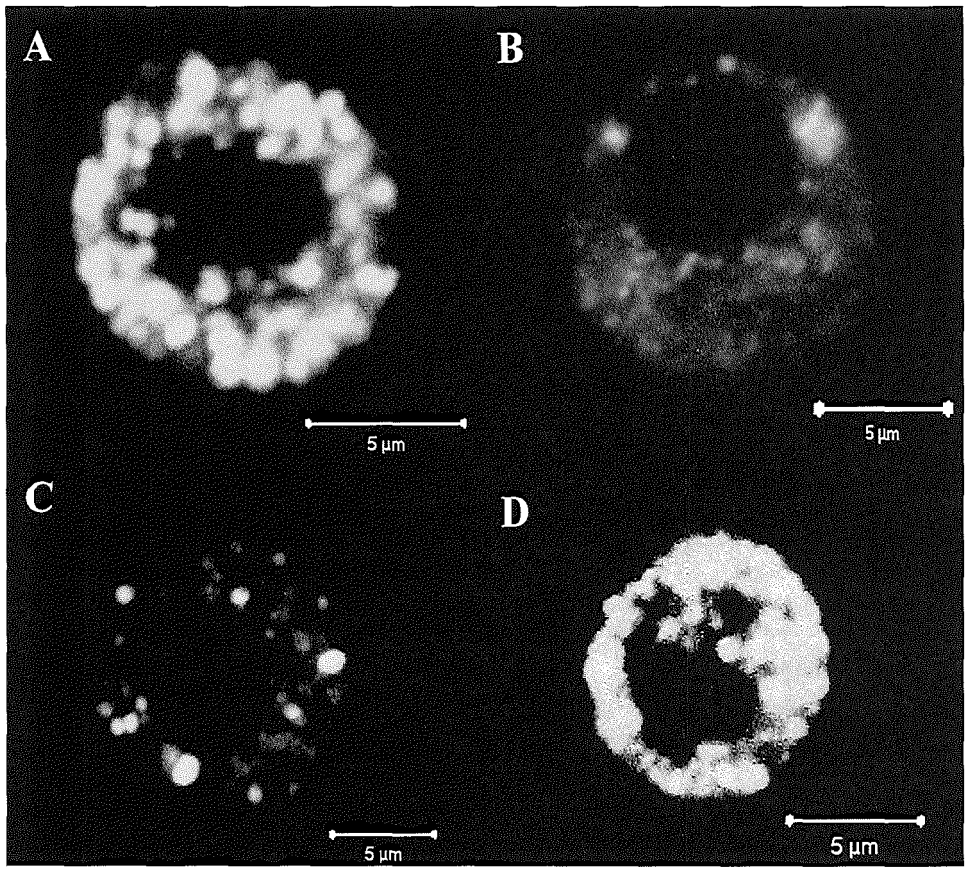

Figure 5. *Localization of cell-associated fluorescence of SP-B_{1-25}*
Mechanically ventilated rats were intratracheally instilled with fluorescent labeled liposomes containing either monomeric SP-B_{1-25} (A, C) or dimeric SP-B_{1-25} (B, D). One hour after instillation alveolar macrophages (C, D) and alveolar type II cells (A, B) were isolated and confocal scans were made. All scans were made using the same microscopic settings.

at physiological amounts it does not affect uptake, though at high concentrations SP-B stimulates the clearance of labeled liposomes. In our laboratory, synthetic analogues of SP-B have been designed, and their function in lowering surface tension has been demonstrated[25]. However, the effects of this synthetic SP-B on the clearance are not yet clarified. In the current study the effect of the incorporation of SP-B analogues on the uptake of labeled liposomes was studied both *in vivo* and *in vitro*.
First, the effects of the synthetic SP-B fragments on the uptake of labeled liposomes were studied *in vitro* and after comparison with nSP-B, it can be concluded that the incorporation of 3% SP-B or SP-B analogues does not influence the uptake by alveolar cells nor the percentage of cells involved in the uptake. However, significant differences between *in vitro* and *in vivo* studies have been demonstrated, and thus similar experiments were performed *in vivo*.
Incorporation of nSP-B in the liposomes did not influence the uptake of these liposomes by

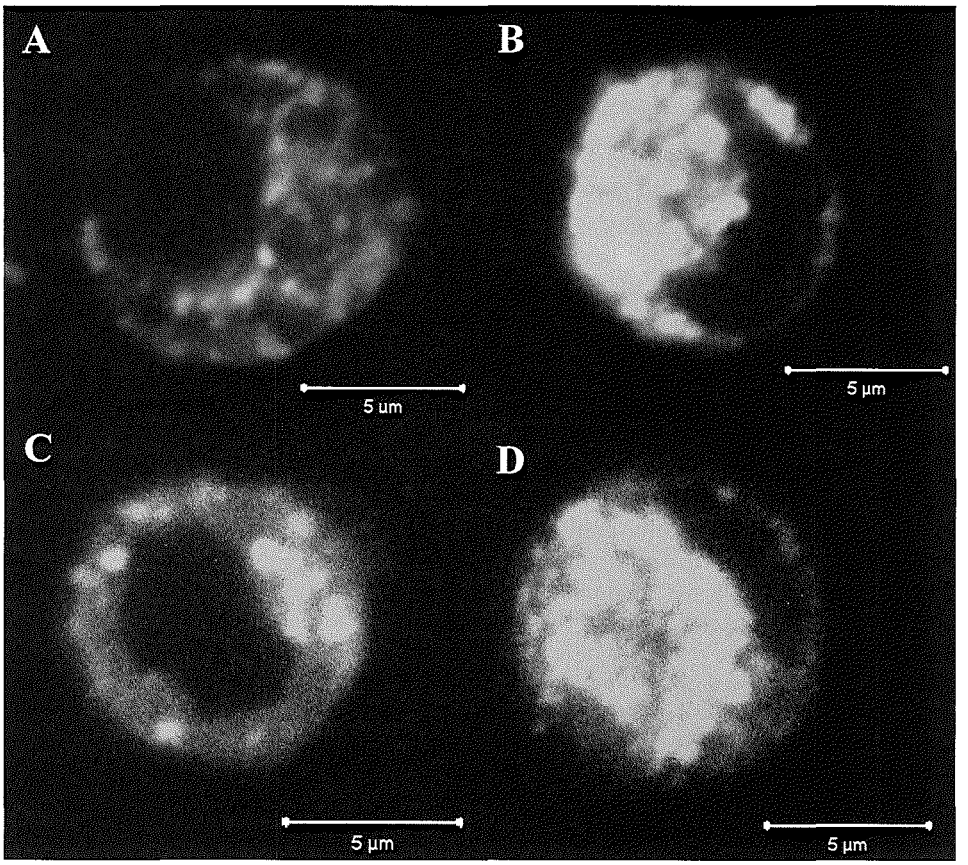

Figure 6. *Localization of cell-associated fluorescence of serine SP-B_{1-25}*
Mechanically ventilated rats were intratracheally instilled with fluorescent labeled liposomes containing either monomeric serine SP-B_{1-25} (A, C) or dimeric serine SP-B_{1-25} (B, D). One hour after instillation alveolar macrophages (C, D) and alveolar type II cells (A, B) were isolated and confocal scans were made. All scans were made using the same microscopic settings.

alveolar type II cells or alveolar macrophages as was expected based on previous studies. Then, with regard to the synthetic SP-B analogues, the inclusion of mSP-B_{1-25} did not influence the uptake of the liposomes by alveolar type II cells, though it did result in an increased uptake of the labeled liposomes by alveolar macrophages. However, the percentage of alveolar type II cells involved in the uptake was significantly increased when mSP-B_{1-25} was incorporated, whereas the percentage of alveolar macrophages, taking up these liposomes was unaffected. Overall, the incorporation of mSP-B_{1-25} increases the uptake of labeled liposomes by alveolar macrophages, whereas the uptake by alveolar type II cells did not differ significantly from the uptake of normal liposomes. The incorporation of dSP-B_{1-25} induced an increased uptake of labeled liposomes by alveolar type II cells, whereas the percentage alveolar type II cells involved in the uptake decreased significantly compared with the normal liposomes and liposomes with SP-B incorporated. The inclusion of dSP-B_{1-25} in the liposome did not affect the uptake per

alveolar macrophage, though the percentage of alveolar macrophages involved in the clearance of these liposomes decreased significantly, resulting in an overall reduced clearance of liposomes with dSP-B_{1-25} in comparison to liposomes without a surfactant protein or with nSP-B.

From these results it can be concluded that surface active analogs of SP-B can influence the contribution of alveolar macrophages on the clearance of surfactant-like liposomes.

These synthetic surfactant proteins, based on human native SP-B were developed with the aim to produce a completely synthetic surfactant. Both mSP-B_{1-25} and dSP-B-1-25 have been demonstrated to improve lung function though dSP-B-1-25 was more efficient than mSP-B-1-25[42, 43] suggesting a legitimate use of these proteins in exogenous surfactant. The results of the current study demonstrate that the use of dSP-B-1-25 is preferable to mSP-B-1-25; the first is not only more surface active[25] but surfactant-like liposomes with dSP-B-1-25 incorporated are also less taken up by alveolar macrophages and thus more liposomes are available for clearance and recycling by alveolar type II cells. Incorporation of mSP-B-1-25 stimulates the uptake of these liposomes by alveolar macrophages, so these liposomes will be more readily removed from the lung.

The two mutant SP-B_{1-25} analogues, serine mSP-B_{1-25} and serine dSP-B_{1-25}, are less surface active and in addition they inhibit the uptake of surfactant-like liposomes as demonstrated in the present study. These results underline the hypothesis that the hydrophobic surfactant proteins, SP-B and SP-C influence the uptake by alveolar macrophages and alveolar type II cells, via interaction with the surfactant lipids. SP-B has been demonstrated to interact with PG, the second major lipid after PC[44, 45]. In addition, this phospholipid has been demonstrated to influence the uptake by alveolar type II cells and alveolar macrophages (submitted data.)[46].

In summary, the current study demonstrates that the more surface active synthetic SP-B analogues do not interfere with the normal surfactant metabolism and can thus be used in exogenous surfactant therapy. In addition, the results underline the hypothesis that the effects of SP-B on the uptake by alveolar cells are mediated by interactions with the surfactant lipids, though an interaction does not necessarily imply an enhanced surface tension lowering activity. Further studies are needed to establish how these synthetic surfactant proteins influence the uptake in diseased animals (as a synthetic exogenous surfactant preparation will be used as a therapeutic intervention for respiratory failure) and their effects on recycling of surfactant.

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Summary

Pulmonary surfactant is essential for normal breathing and disturbance of the surfactant system, caused for example by diseases, is associated with respiratory failure. In order to determine the pathophysiological deviation within the surfactant system, the normal physiology of the surfactant metabolism has to be clear. Because little is known about the uptake of surfactant by alveolar cells even in healthy animals, the studies presented in this thesis are performed using healthy adult animals.

Chapter 1 provides an overview on the uptake of pulmonary surfactant by alveolar type II cells and alveolar macrophages and its regulating factors. First the discovery of and the need for pulmonary surfactant are discussed, followed by a description of the composition of pulmonary surfactant: 90% lipids (dipalmitoylphosphatidylcholine, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, cholesterol) and 10% surfactant specific proteins, SP-A, SP-B, SP-C and SP-D. Subsequently we describe the metabolism of pulmonary surfactant, discuss possible bottlenecks in the metabolism and, more specifically, the need for re-uptake of pulmonary surfactant. This is followed by an analysis of the literature on methods used to measure the uptake, discussing the advantages and disadvantages of the different methods including one developed by our group, as described in **Chapter 2**.

We developed a method using fluorescent labeled liposomes (with a composition similar to natural surfactant) to measure surfactant uptake. Compared with the methods described in the literature, the advantages of our method include: the option to measure both *in vivo* and *in vitro* and to compare the results, as well as demonstrating uptake or intracellular presence rather than cellular adhesion of the fluorescent labeled surfactant-like liposomes. Furthermore, in this method a minor component is used to measure the uptake by replacing it with a similar fluorescent labeled one, and thus the role of the major phospholipids in the regulation of the uptake can be studied, which is discussed in more detail in **Chapter 3**.

Fluorescent labeled liposomes, composed of 86% of the studied phospholipid and 14% of fluorescent labeled phosphatidylethanolamine and cholesterol, were used to investigate the effect of dipalmitoylphosphatidylcholine, phosphatidylcholine, phosphatidylglycerol and phosphatidylinositol *in vitro*. The results showed an increased uptake of negatively charged lipids in comparison to neutrally charged lipids and surfactant-like liposomes. In addition, from competition experiments studying phosphatidylglycerol and dipalmitoylphosphatidylcholine or phosphatidylinositol, it was concluded that the lipids are incorporated by alveolar cells via the same pathway.

In **Chapter 4**, this method is used to determine the effect of the second major phospholipid, phosphatidylglycerol, on the uptake *in vivo*. It was demonstrated that a high concentration of phosphatidylglycerol leads to a decrease of alveolar macrophages in the broncho-alveolar lavage. More interestingly, large amounts of phosphatidylglycerol induce severe deterioration of lung mechanics, as demonstrated by a deterioration of blood gas values. Histological samples showing diffuse alveolar damage support the hypothesis of severe surfactant dysfunction leading to respiratory failure. These results imply a possible fundamental role of phosphatidylglycerol in the surfactant metabolism apart from the surfactant proteins. Although the role of surfactant proteins, especially SP-A, on the uptake of surfactant has been studied extensively, the effects of the hydrophobic proteins SP-B and SP-C on the clearance of surfactant-like liposomes *in vivo* were studied for the first time by our group.

Chapter 5 presents the results of these experiments. The presence of SP-B is unlikely to influence uptake, though inclusion of SP-C in combination with divalent cations and other

solutes is indeed capable of influencing the uptake. These surfactant proteins SP-B and SP-C are nowadays commonly present in therapeutically applied exogenous surfactant. Though exogenous surfactant is used worldwide as a therapeutic life-saving intervention in neonates or infants suffering respiratory distress syndrome, its effect on the endogenous surfactant metabolism is not yet clarified.

Chapter 6 presents a study on the effect of exogenous surfactant on the uptake of surfactant-like liposomes in healthy adult animals. *In vitro*, large surface active surfactant aggregates were demonstrated to increase the uptake of small surface inactive aggregates whereas *in vivo* uptake was decreased by the presence of exogenous surfactant. However, the specific mechanisms underlying these results have to be further elucidated. Because the costs of exogenous surfactant are high and the supply limited, the options to produce a synthetic surfactant are discussed, especially the use of synthetic SP-B analogues. However, these mimics of SP-B should not interfere with the endogenous metabolism and should have a similar function.

Therefore, in **Chapter 7**, the effects of SP-B analogues on the uptake of surfactant-like liposomes are studied. The results demonstrated that, the uptake of liposomes by alveolar type II cells of liposomes with monomeric and dimeric SP-B1-25 (Cys-11>Ala-11) was similar to the uptake of liposomes with native SP-B. However, the incorporation of serine SP-B (Cys-11>Ala-11 and Arg-12>Ser-12), known to have little surface tension lowering activity, resulted in a decreased uptake of these labeled liposomes. The incorporation of SP-B analogues results in an increased uptake of these liposomes by alveolar macrophages for monomeric SP-B-1-25 and serine dimeric SP-B-1-25, whereas dimeric SP-B-1-25 results in a decreased uptake. From these results it was concluded that mutant SP-B peptides, such as serine SP-B, may depress surfactant recycling and thus affect surfactant metabolism.

The work presented in this thesis demonstrates the viability of studying the uptake of surfactant with fluorescent labeled liposomes. The results underline the need to study surfactant uptake both in vivo and in vitro, as well as the influence of composition on uptake, the effect of natural hydrophobic surfactant proteins SP-B and SP-C as well as synthetic SP-B analogues, and finally the influence of exogenous surfactant in healthy animals. It is essential to further explore the uptake of surfactant, for example in diseased animals, to clarify regulating factors and finally develop a disease-specific surfactant, whose composition depends on the underlying disease.

Samenvatting

De aanwezigheid van long surfactant is essentieel voor een normale ademhaling en wordt gewaarborgd via een complex metabolisme. Verstoringen van het surfactant systeem, door ziekten of onrijpe longen, zijn verbonden met respiratoir falen. Om patho-fysiologische afwijkingen binnen het surfactant systeem te kunnen bepalen, is het noodzakelijk dat de normale fysiologie van het surfactant metabolisme bekend is. Over de opname van surfactant door alveolaire cellen is weinig bekend, ook niet in gezonde dieren. Dat is de reden dat de studies zoals beschreven in dit proefschrift uitgevoerd zijn in gezonde dieren. In **Hoofdstuk 1** wordt een overzicht geven van de opname van pulmonaal surfactant door zowel alveolaire type II cellen als alveolaire macrofagen en de regulerende factoren van dit proces. Ten eerste wordt de ontdekking en het belang van surfactant beschreven, wat vervolgens verder gaat in een beschrijving van de chemie van surfactant. Surfactant is opgebouwd uit 90% lipiden (met als belangrijkste: dipalmitoylphosphatidylcholine, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine en cholesterol) en 10% surfactant specifieke eiwitten; SP-A, SP-B, SP-C en SP-D. Daaropvolgend wordt de productie van de componenten of meer algemeen het metabolisme van pulmonaal surfactant beschreven. Mogelijke knelpunten binnen het metabolisme worden aangestipt en de noodzaak van bepaalde onderdelen binnen het complex van surfactant metabolisme benadrukt, in het bijzonder de noodzaak van heropname van pulmonaal surfactant.
Hierna worden de beschikbare methoden op het gebied van het meten van de opname van surfactant en de voor- en nadelen van elk van de methodes beschreven aan de hand van de literatuur, waarna in **Hoofdstuk 2** de methode zoals die ontwikkeld is door onze groep beschreven wordt.
Deze bestaat uit het meten van de opname van lipiden door alveolaire cellen met behulp van fluorescent gelabelde liposomes en flow-cytometrie. Deze methode, waarbij cel-geassocieerde fluorescentie gebruikt wordt als maat voor de opname van fluorescent gelabelde liposomen, heeft een aantal voordelen ten opzichte van andere methoden (bijvoorbeeld radioactiviteit): zoals de mogelijkheid om zowel *in vivo* als *in vitro* te meten en deze resultaten met elkaar te vergelijken, alsook het aan tonen dat de fluorescentie wordt veroorzaakt door intracellulaire aanwezigheid en niet door 'kleven' aan het celmembraan. Bovendien maken de meeste methoden gebruik van gelabeld dipalmitoylphosphatidylcholine, een van de meest essentiële lipiden, terwijl onze methode een andere lipid gebruikt als tracer, namelijk phosphatidylethanolamine een lipid dat slechts in geringe mate voorkomt in pulmonaal surfactant (8 gewichtsprocenten). Het labelen van een niet-essentieel lipid maakt het mogelijk de rol van 'essentiële' lipiden te onderzoeken.
In **Hoofdstuk 3** wordt beschreven hoe met behulp van gemodificeerde liposomen, die voor 86% bestaand uit het te bestuderen lipid, fluorescent gelabeld phosphatidylethanolamine en cholesterol, het effect van dipalmitoylphosphatidylcholine, phosphatidylcholine, phosphatidylglycerol en phosphatidylinositol bepaald kan worden. Uit de resultaten van deze experimenten kan geconcludeerd worden dat negatief geladen lipiden beter/sneller opgenomen worden dan neutraal geladen lipden of surfactant-achtige liposomen. Daarnaast wezen competitie experimenten met phosphatidylglycerol en dipalmitoylphosphatidylcholine of phosphatidylinositol aan dat deze lipiden opgenomen worden via dezelfde weg, echter dat er een voorkeur bestaat voor 'negatief' geladen lipiden.
In **Hoofdstuk 4** wordt dit verder onderzocht door het effect van phosphatidylglycerol, het op 2 na belangrijkste lipid, *in vivo* te bestuderen. Daaruit kwam naar voren dat hoge concentraties phosphatidylglycerol leiden tot een afname in het aantal alveolaire macrofagen in broncho-

alveolaire lavage. Tevens en niet geheel onbelangrijk, induceert een verhoogde concentratie phosphatidylglycerol een verslechtering van de arteriële oxygenatie als ook diffuse alveolaire schade zoals op histologische coupes aangetoond werd. Het dient opgemerkt te worden dat de aanwezigheid van co-factoren, als calcium en magnesium, in het liposoom deze negatieve effecten van phosphatidylglycerol kan voorkomen. Hiermee is aangetoond dat naast de in de literatuur beschreven surfactant specifieke eiwitten, met name SP-A, de lipid samenstelling eveneens een rol speelt in de regulatie van de opname van surfactant, in het bijzonder phosphatidylglycerol. Echter, de rol van de hydrofobe eiwitten op de opname van surfactant *in vivo* is niet bekend.

In **Hoofdstuk 5** worden de resultaten beschreven van de experimenten zoals die uitgevoerd zijn om de rol van SP-B en/of SP-C op de heropname van surfactant te bestuderen. Het hydrofobe SP-B, essentieel voor de surfactant functie aangezien afwezigheid bij geboorte leidt tot directe dood, lijkt geen effect op de opname van surfactant te hebben, SP-C daarentegen, voornamelijk in de aanwezigheid van eerder genoemde factoren als calcium en magnesium, kan de opname daadwerkelijk beïnvloeden. Deze twee surfactant eiwitten, SP-B en SP-C, worden heden ten dage toegevoegd aan exogeen surfactant, zoals dat gebruikt wordt in klinische toepassing. Ofschoon exogeen surfactant therapie wereldwijd geaccepteerd is als een levensreddende interventie in neonaten en kinderen leidend aan Respiratory Distress Syndrome (RDS), ongeacht de oorzaak, zijn de effecten van exogeen surfactant op het endogene surfactant metabolisme nog niet geheel opgehelderd.

In **Hoofdstuk 6** van dit proefschrift wordt het effect van exogeen surfactant op de opname van op surfactant lijkende liposomen in gezonde dieren bestudeerd, alsook *in vitro*. In deze laatste situaties werd aangetoond dat de grote, oppervlakte-actieve aggregaten van surfactant de opname van fluorescent gelabelde kleine aggregaten verhoogd. Aan de andere kant, *in vivo*, leidde de aanwezigheid van exogeen surfactant tot een verlaging van de opname van liposomen. De onderliggende mechanismen van deze tegenstrijdige effecten van exogeen surfactant op de opname zullen verder onderzocht moeten worden, ofschoon duidelijk is dat 'omgevingsfactoren' een rol spelen.

De kosten van exogeen surfactant zijn hoog en derhalve wordt er gezocht naar een mogelijkheid om een synthetisch surfactant, alsook synthetische varianten/mimics van SP-B te produceren. Echter, deze synthetische peptiden mogen het endogene metabolisme logischerwijs niet beïnvloeden.

Recent, zijn een aantal SP-B analogen ontwikkeld en in **Hoofdstuk 7** wordt hun effect op de opname van surfactant-achtige liposomen bestudeert en beschreven dat monomerisch en dimerisch SP-B-1-25 de opname door alveolaire type II cellen niet beïnvloed, maar het vervangen van aminozuren door serine leid tot de 'serine' variant van SP-B-1-25 en geeft een verminderde opname door type II cellen. Met het oog op alveolaire macrofagen valt op dat de aanwezigheid van monomerisch SP-B-1-25 of serine dimerisch SP-B-1-25 leidt tot een verhoogde opname van de liposomen; de aanwezigheid van dimerisch SP-B-1-25 in het liposoom daarentegen leidt tot een verminderde opname van deze liposomen door alveolaire macrofagen. Uit deze resultaten kan geconcludeerd worden dat mutant SP-B peptide, zoals de 'serine variant', de surfactant recycling kan verminderen en dus een effect zou kunnen hebben op het endogene surfactant metabolisme.

Tot slot kan uit de resultaten zoals beschreven in dit proefschrift geconcludeerd worden dat het gebruik van fluorescent gelabelde liposomen een goede methode is om surfactant opname door alveolaire cellen te meten met een aantal voordelen ten opzichte van de tot op heden gebruikte methoden. Met behulp van deze methode is aangetoond dat er essentiële verschillen bestaan tussen de *in vivo* en *in vitro* situatie en dat de opname van surfactant niet alleen bepaald wordt door surfactant specifieke eiwitten maar zeker ook door haar lipid samenstelling. Ten aanzien van het effect van exogeen surfactant op de opname en mogelijk dus het endogene metabo-

lisme moet aangestipt worden dat er *in vitro* een stimulatie van de opname gezien werd terwijl *in vivo* de opname verminderde; echter de onderliggende mechanismen hiervan zijn niet bekend. Met het oog op de ontwikkeling van een synthetisch surfactant moet gemeld worden dat het gebruik van surfactant eiwit analogen mogelijk is, ofschoon variaties in aminozuren zouden kunnen leiden tot een verandert metabolisme van het specifieke eiwit, zoals aangetoond voor de varianten, c.q. synthetische analogen van SP-B.
Het werk dat gepresenteerd wordt in dit proefschrift laat zien dat de opname van surfactant gemeten kan worden met fluorescent gelabelde liposomen; en onderstreept de noodzaak om zowel *in vivo* als *in vitro* te meten, laat een effect van samenstelling op de opname zien, demonstreert het effect van de natuurlijk hydrofobe eiwitten SP-B en SP-C als ook van synthetische SP-B analogen op de opname en tot slot het effect van exogeen surfactant op de opname in gezonde dieren. Het is essentieel om de opname van surfactant verder te onderzoeken, bijvoorbeeld in zieke dieren, met het oog op opheldering van regulerende factoren en uiteindelijk de ontwikkeling van een ziekte specifiek surfactant, waarbij haar samenstelling afhankelijk is van het onderliggend lijden.

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Curriculum vitae

De schrijver van dit proefschrift, Davey Poelma, werd geboren op 23 mei 1977 in Beek. In 1995 rondde hij zijn middelbare school af met het behalen van het VWO-diploma op Scholengemeenschap St. Michiel te Geleen. Datzelfde jaar begon hij de studie Geneeskunde aan de Erasmus Universiteit Rotterdam, alwaar hij in gedurende de laatste periode van zijn studie in contact kwam met Prof. dr. B. Lachmann. Na het behalen van zijn doctoraal examen in 2000, was hij werkzaam als assistent in opleiding (AIO) op de afdeling Experimentele Anesthesiologie, alwaar hij wetenschappelijk onderzoek deed welk uitmondde in de studies zoals beschreven in dit proefschrift onder begeleiding van Dr. J.F. van Iwaarden en Prof. dr. B. Lachmann. Tijdens deze periode onderhield hij nauwe samenwerking met de afdeling kindergeneeskunde aan de Erasmus Universiteit Rotterdam en later met de afdeling Neonatologie aan het Academisch Ziekenhuis Maastricht. In oktober 2004 zal hij beginnen met het laatste stadium van zijn opleiding geneeskunde, en instromen in de co-assistentschappen.